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(71) Applicant (for all designated States except US): CONNAUGHT LABORATORIES LIMITED [CA/CA]; 1755 Steeles Avenue, North York, Ontario M2R 3T4 (CA).

(72) Inventors; and

- (75) Inventors/Applicants (for US only): SOKOLL, Kenneth, K. [CA/CA]; 43 Dod's Drive, Alton, Ontario LON 1A0 (CA). CHONG, Pele [CA/CA]; 32 Estoril Street, Richmond Hill, Ontario L4C 0B6 (CA). KLEIN, Michel, H. [CA/CA]; 16 Munro Boulevard, Willowdale, Ontario M2P 1B9 (CA).
- (74) Agent: STEWART, Michael, I.; Sim & McBurney, 6th floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).

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(54) Title: BIODEGRADABLE TARGETABLE MICROPARTICLE DELIVERY SYSTEM

(57) Abstract

Copolymers designed for use as particulate carriers containing functionalizable amino acid subunits for coupling with targeting ligands are described. The copolymers are polyesters composed of α -hydroxy acid subunits such as D,L-lactide and pseudo- α -amino acid subunits which may be derived from serine or terpolymers of D,L-lactide and glycolide and pseudo- α -amino acid subunits which may be derived from serine. Stable vaccine preparations useful as delayed release formulations containing antigen or antigens and adjuvants encapsulated within or physically mixed with polymeric microparticles are described. The particulate carriers are useful for delivering agents to the immune system of a subject by mucosal or parenteral routes to produce immune responses, including antibody and protective responses.

Poly-D,L-Lactide-co-Glycolide-co-pseudo-L-Z-Serine Ester (PLGpZS)

Poly-D,L-Lactide-co-Glycolide-co-pseudo-L-Serine Ester (PLGpS)

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Copolymers designed for use as particulate carriers containing functionalizable amino acid subunits for coupling with targeting ligands are described. The copolymers are polyesters composed of α -hydroxy acid subunits such as D,L-lactide and pseudo- α -amino acid subunits which may be derived from serine or terpolymers of D,L-lactide and glycolide and pseudo- α -amino acid subunits which may be derived from serine. Stable vaccine preparations useful as delayed release formulations containing antigen or antigens and adjuvants encapsulated within or physically mixed with polymeric microparticles are described. The particulate carriers are useful for delivering agents to the immune system of a subject by mucosal or parenteral routes to produce immune responses, including antibody and protective responses.

Poly-D,L-Lactide-co-Glycolide-co-pseudo-L-Z-Serine Ester (PLGpZS)

HBr/AcOH

$$H = \begin{pmatrix} CH_3 & O & O \\ O & V & O$$

Poly-D,L-Lactide-co-Glycolide-co-pseudo-L-Serine Ester (PLGpS)

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WO 98/28357 PCT/CA97/00980

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TITLE OF THE INVENTION

BIODEGRADABLE TARGETABLE MICROPARTICLE DELIVERY SYSTEM

FIELD OF THE INVENTION

The present invention relates to biodegradable microparticles for delivery of a biologically active material and is particularly concerned with such microparticles that are targetable to particular cell types.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States Patent No. 08/770,050 filed December 20, 1996.

BACKGROUND OF THE INVENTION

Vaccines have been used for many years to protect humans and animals against a wide variety of infectious diseases. Such conventional vaccines consist of attenuated pathogens (for example, polio virus), killed pathogens (for example, Bordetella pertussis) or immunogenic components of the pathogen (for example, diphtheria toxoid and hepatitis B surface antigen).

Some antigens are highly immunogenic and are capable alone of eliciting protective immune responses. Other antigens, however, fail to induce a protective immune response or induce only a weak immune response. The immune response of a weakly immunogenic antigen can be significantly enhanced if the antigens are co-administered with adjuvants. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system.

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Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses. Adjuvants have been identified that enhance the immune response to antigens delivered parenterally.

Adjuvants are commonly employed with antigen in vaccine formulations whereby the induction of systemic immunity through parenteral immunization (intramuscular subcutaneous) or is obtained. This approach suitable for infectious agents gaining access to the body via damaged skin (i.e. Tetanus), however, there problems encountered due to side-effects associated toxicity of many adjuvants administered in fashion. Only those vaccines formulated from aluminum salts (aluminum phosphate or hydroxide) find routine use in human and veterinary However, even these adjuvants are not vaccination. suitable for use with all antigens and can also cause irritation at the site of injection. There is a clear need to develop adjuvants which safely enhance the immunogenicity of antigens at the site of injection.

There are other problems specific to the nature of the antigen being used. For example, most conventional non-living vaccines require multiple doses for effective immunization. Live attenuated vaccines and many nonliving liquid vaccines suffer from the need for controlled storage conditions and are susceptible to inactivation (e.g. thermal sensitivity). also problems associated with combining vaccines in single dosage forms, due to adjuvant incompatibilities, pH, buffer type and the presence of salts.

Mucosal immunity is induced primarily by induction of secretory immunoglobulin (sIgA) in intestinal, bronchial or nasal washings and other external

For example, parenteral cholera vaccines secretions. have been shown to offer limited protection whereas the more recently developed oral form is highly effective 1 - throughout this specification, references are referred to in parenthesis to more fully 5 describe the state of the art to which this invention Full bibliographic information for each pertains. citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference 10 into the present disclosure). Studies with human volunteers have shown that oral administration - of influenza vaccine is effective at inducing secretory anti-influenza antibodies in nasal secretions and substances have been identified which might be useful 15 as adjuvants for such ingested vaccines. However, most of these adjuvants are relatively poor in terms of improving immune responses to ingested Currently, most of these adjuvants have been determined 20 safe and efficacious in enhancing responses in humans and animals to antigens that are administered via the orogastrointestinal, nasopharyngeal-respiratory and genital tracts or in the ocular orbits. However, administration of antigens via these routes is generally ineffective in eliciting an 25 response. Although the above example illustrates the potential of these immunization modes, the development of vaccine formulations for use these routes has been slow for various reasons. The 30 inability to immunize at the mucosal generally believed to be due to include:

(i) antigen degradation via the acid and/or proteolytic enzymes present during the transit to the mucosal surfaces;

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- (ii) antigen degradation by secretions presented
 at the mucosal epithelium;
- (iii) limited adsorption across the mucosal
 epithelium;
- 5 (iv) the dilution of the antigen to a concentration that is below that required to induce immune responses; and
 - (v) ineffective adjuvants and/or delivery
 systems.

The problems associated with the use of adjuvants in parenteral vaccine formulations and the lack of suitable systems for vaccine delivery to mucosal sites understates the need for new techniques that are effective when administered by various routes and are inherently free from associated toxicity concerns or side-effects.

It is also desired to provide vaccine delivery in a single dosage form for both human and animal immunizations as this has the advantage of reducing time and cost, and in human medicine, increases patient compliance which is of extreme importance in developing countries where access is restricted. This is especially true for infants within these countries.

order to increase immune responses administered antigens, a carrier may be used to protect the antigen from degradation and also modulate the uptake of these materials in vivo. Sensitive antigens may be entrapped to protect them against destruction, reduction in immunogenicity or dilution. Methods for formulating a carrier include dispersing an antigen within a polymeric matrix (monolithic matrix) or by the coating of a polymeric material around an antigen to give an outer protective wall (core-shell). manipulation of the formulation protocol can allow for

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control over the average size of these materials. This has been shown to be important for the uptake of particulates via oral delivery at specialized M-cells of the Peyers patches within the intestinal tract.

5 U.S. Patent No. 5,151,264 describes a particulate phospholipid/glycolipid/polysaccharide carrier nature that has been termed Bio Vecteurs Moleculairs (BVSM). The particulate carriers intended to transport a variety of molecules having biological activity in one of the layers thereof. 10 However, U.S. Patent No. 5,151,264 does not describe particulate carriers containing antigens immunization particularly and does not describe carriers particulate for immunization via 15 orogastrointestinal, nasapharyngeal-respiratory urogenital tracts and in the ocular orbits or other mucosal sites.

Eldridge et al. (refs 2 and 3) observed the delayed release antigen in vivo from biodegradable microspheres manufactured from polylactide-coglycolide copolymer also known as PLG or PLGA. Numerous other polymers have been used to encapsulate antigens for formulation into microparticles and some of these include polyglycolide, polylactide, polycaprolactone, polyanhydrides, polyorthoesters and poly(αhydroxybutyric acid).

U.S. Patent No. 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactide-co-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone),

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poly(esteramides), polyorthoesters and poly(α hydroxybutyric acid), and poly anhydrides. The encapsulated antigen was administered to mice via gastric intubation and resulted in the appearance of significant antigen-specific IgA antibodies in saliva and gut secretions and in sera. As is stated in this patent, in contrast, the oral administration of the same amount of unencapsulated antigen was ineffective at inducing specific antibodies of any isotype in any of the fluids tested. Poly(DL-lactide-co-glycolide) microcapsules were also used to administer antigen by parenteral injection.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive microspheres and antigenic vaccine ingredients. microspheres being of starch, gelatin, collagen or delivery vehicle albumin. This particularly intended for the uptake of vaccine across the nasal mucosa. , The deliverv vehicle additionally contain an absorption enhancer. antigens are typically encapsulated within protective polymeric materials.

U.S. Patent No. 5,571,531 describes particulate carriers comprising a solid matrix of a polysaccharide and a proteinaceous material. A functionalized silicone polymer is bonded to the matrix for the delivery of materials having biological activity.

Although time-delayed release of antigen was shown in the above work, difficulties were encountered when microparticles are manufactured by the described methods. The exposure of biological materials to the organic solvents and physical forces used can lead to denaturation. It may be also be difficult to scale-up

the procedures. Furthermore, hydrophilic antigens may be inefficiently encapsulated.

It would be desirable to provide improved carriers without such limitations. It would be particularly desirable to provide polymeric materials which can be formulated into microparticles and microspheres and which contain targeting moieties to target the antigen to preselected ligands. This would have tremendous potential for cells of the immune system.

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SUMMARY OF THE INVENTION

The present invention is directed towards the production of a novel and useful polymer that has properties suitable for manufacturing by various processes into microparticles and microspheres. In this invention, modifications of existing processing procedures results in significant improvement in encapsulation efficiencies.

This invention is further directed to the production of useful vaccine delivery systems for antigen(s) or antigen and co-adjuvant cocktails by various immunization routes which include parenteral, oral and intranasal.

In accordance with a first aspect of the invention, there is provided a novel biodegradable, biocompatible polymer, including those having a molecular weight of about 5,000 to about 40,000 daltons, having a backbone of the general formula:

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wherein:

 R_1 , R_2 , R_3 , R_4 and R_5 are selected independently and are selected from H, linear or branched alkyl groups;

R₆ is selected from H, an amine protecting group, a spacer molecule or a biologically active species;

X is selected from an O or S group; and x and y are integers, including values such that at least about 95% of the polymer is comprised of α -hydroxy acid residues.

The novel polymers are derived by copolymerization of monomers comprising at least one α -hydroxy acid or derivative thereof, including cyclic divesters and at least one pseudo- α -amino acid. The α -hydroxy acids are generally of the formula $R_1R_2COHCO_2H$, where the R_1 and R_2 groups are H, linear or branched alkyl groups. The α -hydroxy acids may comprise a mixture of α -hydroxy acids, at least one of the mixture of α -hydroxy acids having R_1 and R_2 groups which are hydrogen and another α -hydroxy acid having an R_1 group which is CH_3 and R_2 which is H. The pseudo- α -amino acids are generally of the formula $R_5CHNHR_6CO_2H$, where the R_5 group is a hydroxyl methyl or methyl thiol group and R_6 is an amine protecting group.

The amine protecting groups may be carbobenzyloxy, benzyl, paramethoxybenzyl, benzyloxymethoxy, tert-butyloxycarbonyl or [9-fluorenylmethyloxy]carbonyl.

The α -hydroxy acids are generally selected from L-30 lactic acid, D,L-lactic acid, glycolic acid, hydroxy valeric acid and hydroxybutyric acid. The at least one pseudo- α -amino acid may be serine.

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In a preferred aspect of the invention, the polymers are poly-D,L-lactide-co-glycolide-co-pseudo-Z-serine ester (PLGpZS) and poly-P,L-lactide-co-glycolide-co-pseudo-serine ester (PLGpS).

The polymers may contain biologically active moieties, such as cell bioadhesion groups, macrophage stimulators, polyethylene glycol, poly amino acids and/or protected amino acid residues, covalently bound to the polymer directly or through side groups.

preferred embodiment, the bioactive substituents are linked to the polymer via the amino groups on the amino acid moieties directly or via a suitable spacer molecule. The spacer molecule can be selected from α -hydroxy acids represented by the formula R7R8COHCO2H, where R₇ or R₈ groups are independently selected from H, linear or branched alkyl units and α -amino acids represented by the formula R₉CHNHR₁₀CO₂H, where the R₉ group is a hydroxyl methyl: or methyl thiol group and R_{10} is an amine protecting group.

In accordance with a further aspect of the invention, the invention provides a method of making a biodegradable, biocompatible polyester, which comprises co-polymerizing at least one α -hydroxy acid and at least one pseudo- α -amino acid.

In accordance with another aspect of the present invention, there is provided a process for making a biodegradable, biocompatible polymer of the general formula provided herein which comprises forming a mixture of monomers comprising at least one α -hydroxy acid and at least one pseudo- α -amino acid with an organic solvent solution of an esterification catalyst under inert atmospheric conditions, copolymerizing the

monomers and isolating the resultant polymer. The catalyst used is preferably stannous 2-ethylhexanoate.

The polymer formed by the process can be further deprotected by solid phase catalytic reduction or alternatively by acid catalysis using hydrogen bromide in acetic acid solution.

The process can also further comprise forming the polymer into a film or microparticles.

In accordance with another aspect of this invention, there is provided a particulate carrier for the delivery of biologically active materials to a host, the carrier comprising a polymer, including those having a molecular weight of about 5,000 to about 40,000 daltons, having the general formula:

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wherein;

 R_1 , R_2 , R_3 , R_4 and R_5 are selected independently and are selected from H, linear or branched alkyl groups;

R₆ is selected from H, an amine protecting group, a spacer molecule or a biologically active species;

X is selected from an O or S group; and

x and y are integers, including values such that at least about 95% of the polymer is comprised of $\alpha-$ hydroxy acid residues.

The particulate carrier generally has a particle size of about 1 to 10 μM_{\cdot}

In a further aspect of the present invention is a process for making a particulate carrier for the

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delivery of at least one biologically active material to a host, the process comprising:

- (a) mixing an organic solvent phase comprising an α -hydroxy acid polymer or copolymer with an aqueous composition comprising dispersed or dissolved biologically active material to form a first water-in-oil emulsion;
- (b) dispersing the first water-in-oil emulsion into an aqueous detergent phase to form a second water-in-oil-in-water double emulsion;
- (C) removing water from the second double emulsion to form microspheres; and
- (d) collecting the microspheres and having the biological material entrapped therein.

15 The particulate carrier of the present invention can be used as a composition having a biologically active material mixed therewith or entrapped within. The biological materials used may be selected from those which elicit an immune response. Such materials may comprise Haemophilus influenzae proteins, 20 a non-proteolytic Hin-47 analog, D15, P1, P2, and P6. The biologically-active material may comprise at least one influenza virus, which may be a multivalent or monovalent influenza virus vaccine, or influenza virus 25 protein, such as an influenza virus monovalent protein vaccine. In addition, the biologically-active material comprise at least one Moraxella catarrhalis protein, such as the Tbp2 protein of M. catarrhalis. further biologically-active material which 30 employed may be at least one Helicobacter pylori protein, such as Urease. Other biological material may include proteins, protein mimetics, bacteria, bacterial lysates, viruses (e.g. respiratory syncytial virus), virus-infected cell lysates, DNA plasmids, antisense

RNA, peptides (e.g. CLTB-36 and M2), antigens, antibodies, pharmacological agents, antibiotics, carbohydrates, lipids, lipidated amino acids tripalmitoyl cysteine), glycolipids, haptens combinations and mixtures thereof.

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The first water-in-oil emulsion may additionally comprise at least one organic solvent soluble adjuvant, which may be lipophilic. Such organic solvent adjuvant may be selected from the group consisting of BAY R1-005, tripalmitoyl cysteine and DC-chol. The presence of lipophilic moiety serves to increase encapsulation efficiency and to protect the antigen and release during formulation and enables particles to present antigen to the immune system more efficiently than traditional formulation and hence provides a more efficacious vaccine.

The first water-in-oil emulsion also may additionally comprise at least one water adjuvant, which may be a polymeric water soluble adjuvant, such as PCPP or a mucosal adjuvant, such as CT-X or subunit thereof or LT. The presence of the water soluble adjuvant serves to increase encapsulation efficiency of the process and protects the antigen during formulation and release and prevents the antigen to the immune system more efficiently than traditional microparticle formulation. thereby providing a more efficancious vaccine.

The present invention also provides an immunogenic composition comprising the particulate carrier provided herein and physiologically acceptable a therefor. composition can The be administered mucosally or parenterally. The immune response is an antibody response which is a local or serum antibody In accordance with this aspect of response.

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invention, there is provided a controlled or delayed release vaccine preparation in stable particulate form and a method of making such a vaccine preparation. The particles are microspherical and contain a matrix of biodegradable polymer and antigen(s) and/or antigen plus co-adjuvant containing regions.

Advantages of the invention include:

- (a) fully biodegradable and biocompatible microparticle formulation;
- (b) facilitated antigen presentation to the cells of the immune system resulting in improved antigen immunogenicity;
- (c) improved formulating conditions which increase the bioavailability of the antigen.

Additional embodiments of the present invention include the use of the particulate carrier in diagnostic assays and for therapeutic strategies.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 is a schematic showing the ring opening polymerization (ROP) of lactic acid dimer with glycolic acid dimer and N-(carbobenzyloxy)-L-serine lactone with subsequent deprotection in accordance with a preferred aspect of the present invention.

Figure 2 is a schematic showing the attachment of biologically active moieties to polymer through the side chain of the α -amino acid sub-unit within the polymer. Representative targeting groups include polyethylene glycol (PEG) for water solubility and circulation, macrophage stimulators and cell

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bioadhesion groups. Spacer ligands derived from α -hydroxy acids or α -amino acids may be incorporated to facilitate attachment of the bioactive ligand.

Figure 3 is a schematic detailing the process used produce microparticles in accordance embodiment of the invention. In this figure, Hin-47 is a non-proteolytic recombinant protein analog derived Haemophilus influenzae (as from described in Patent No. 5,506,139), Flu X31 is influenza strain X31 or A-Texas, rD-15 is recombinant protein derived from Haemophilus influenzae (as described in WO 94/12641), PVA = poly vinyl alcohol. Flu(tri) is trivalent flu, catarrhalis transferrin Moraxella protein 2 and rUrease is recombinant Helicobacter pylori urease.

Figure 4 shows a typical size distribution for poly-D,L-lactide-co-glycolide-co-pseudo-(Z)-serine ester (PLGpZS) and poly-D,L-lactide-co-glycolide-co-pseudo-serine ester (PLGpS) microparticles when prepared in the presence of PBS or a typical protein (non-proteolytic Hin-47 analog) as determined by laser diffraction measurements.

Figure 5 shows a scanning electron micrograph of microparticles prepared from poly-D,L-lactide-co-glycolide-co-pseudo-(Z)-serine ester (PLGpZS) and poly-D,L-lactide-co-glycolide-co-pseudo-serine ester (PLGpS) in the presence of phosphate buffered saline(PBS).

Figure 6 shows a scanning electron micrograph of microparticles prepared from poly-D,L-lactide-co-glycolide-co-pseudo-(Z)-serine ester (PLGpZS) and poly-D,L-lactide-co-glycolide-co-pseudo-serine ester (PLGpS) in the presence of a typical antigen/PBS mixture such as Hin-47/PBS.

Figure 7A shows the *in vitro* release profile for non-proteolytic Hin-47 analog encapsulated within PLG, PLGpZS and PLGpS microparticles over a three month period obtained from 14 mg samples (typical core loadings range from 2.5 to 5.7 μ g protein/mg of microparticles) that were incubated in PBS (pH = 7.4) and maintained at 37°C.

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Figure 7B compares the % cumulative release of non-proteolytic Hin-47 analog from PLG, PLGpZS and PLGpS microparticles over a three month period obtained from ~14 mg samples (typical core loadings range from 2.5 to 5.7 μ g protein/mg of microparticles) that were incubated in PBS (pH = 7.4) at maintained and 37°C.

Figure 8A shows the serum IqG responses in mice. immunized subcutaneously (S.C.) following immunization protocols by the 47 kDa membrane protein from Haemophilus influenzae (non-proteolytic Hin-47) analog). Groups of 5 mice were immunized on days 1 and 35 with 250 μL of PBS, pH 7.4, containing either 0.2 or 0.6 µg of non-proteolytic Hin-47 analog incorporated PLGpZS into PLG, or PLGpS microparticles. obtained on days +10, +24, +35, +46 and +60 were for evaluated the presence of anti-Hin-47 antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figure 8B shows the serum IgG responses in mice immunized subcutaneously (S.C.) following various immunization protocols by the 47 kDa membrane protein from Haemophilus influenzae (non-proteolytic Hin-47 analog). Groups of 5 mice were immunized on days 1 and 35 with 250 μ L of PBS, pH 7.4, containing either 0.8 or 2.5 μ g of non-proteolytic Hin-47 analog physically mixed with PLG microparticles. Sera obtained on days +10, +24, +35, +46 and +60 were evaluated for the

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presence of anti-Hin-47 IgG antibodies using an enzymelinked immunosorbent assay (ELISA).

Figure 8C shows the serum IgG response subtype profile for pooled bleeds obtained on days +35 and +60 from the study conducted as described in Figure 8A and 8B.

Figure 9A shows the IgG serum antibody responses in mice immunized intragastrically (I.G.) by the 47 kDa membrane protein from Haemophilus influenzae (non-proteolytic Hin-47 analog). Groups of 5 mice were immunized on days 1, 7, 14 and 57 with 500 µL of PBS, pH 7.4, containing 4 µg of Hin-47 analog incorporated into PLG, PLGpZS or PLGpS microparticles or physically mixed with PLG, PLGpZS or PLGpS microparticles. Sera obtained on days +13, +35, +56 and +78 were evaluated for the presence of anti-Hin-47 IgG antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figure 9B shows the serum IgG response subtype profile for pooled bleeds obtained on days +56 and +78 from the study conducted as illustrated in Figure 9A.

Figure 9C shows the serum IgA response for the bleed obtained on day +78 from the study conducted as illustrated in Figure 9A.

Figure 10A shows the IgG serum antibody responses in mice immunized intranasally (I.N.) by a (1:1)cocktail of the 47 kDa membrane protein Haemophilus influenzae (non-proteolytic Hin-47 analog) and the 115 kDa membrane protein from Haemophilus influenzae (rD-15). Groups of 5 mice were immunized on days 1, 7, 14 and 57 with 25 μ L of PBS, pH 7.4, containing 4 µg of non-proteolytic Hin-47 incorporated into PLG, PLGpZS or PLGpS microparticles physically mixed with PLG, PLGpZS microparticles. Sera obtained on days +13, +35, +56

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and +78 were evaluated for the presence of anti-Hin-47 IgG antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figure 10B shows the serum IgG response subtype profile for pooled bleeds obtained on days +56 and +78 from the study conducted as described in Figure 10A.

Figure 10C shows the serum IgA response for the bleed obtained on day +78 from the study conducted as described in Figure 10A.

Figure 10D shows the lung lavage IgG response obtained on day +78 from the study conducted as described in Figure 10A.

Figure 10E shows the lung lavage sIgA response obtained on day +78 from the study conducted as described in Figure 10A.

Figure 11 shows the anti-Flu X31 (i.e. influenza virus type A strain X31) IgG serum antibody responses following various immunization protocols. Groups of 6 mice were immunized subcutaneously (S.C.) on day 1 with 250 μ L of PBS, pH 7.4, containing 1.5 μ g of HA incorporated into PLG, PLGpZS or PLGpS microparticles. Sera obtained on days +21 and +33 and were evaluated for the presence of anti-Flu X-31 IgG antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figure 12 shows the anti-Flu X31 (i.e. influenza virus type A strain X31) IgG serum antibody responses following various immunization protocols. Groups of 6 mice were immunized intranasally (I.N.) on days 1 and 34 with 25 µL of PBS, pH 7.4, containing 1.5 µg of HA incorporated into PLG, PLGpZS or PLGpS microparticles. Sera obtained on days +21, +33, +57, +78 and +92 were evaluated for the presence of anti-Flu X-31 IgG antibodies using an enzyme-linked immunosorbent assay (ELISA).

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Figure 13 shows the hemagglutination inhibition antibody assay (i.e. influenza virus strain A-Texas) responses for pooled sera (days +21 and +42 or +57) following a single dose subcutaneous administration. Groups of 6 mice were immunized subcutaneously (S.C.) On day 1 with 250 μl of PBS, pH 7.4, containing either 0.35 μg of HA or 3.5 μg of HA incorporated into PLGpS microparticles or 0.35 μg of HA and ~2 μg of BAY R1-005 or 3.5 µg of HA and ~20 µg of BAY R1-005 incorporated into PLGpS microparticles or 0.35 μg of HA or 3.5 μg of 10 HA physically mixed with PLGpS microparticles or 0.35 μg of HA and 2 μg of BAY R1-005 or 3.5 μg of HA and 20 of BAY R1-005 physically mixed with microparticles. Sera obtained on days +21 and +42 were evaluated for the inhibition of hemagglutination of 15 erythrocytes.

Figures 14a to c show the anti-Flu (trivalent) IgG serum antibody responses (for each influenza virus strain contained in trivalent vaccine; A/Texas (Figures 14a), A/Johannesburg (Figure 14b) and B/Harbin Figure 14C)) following various immunization protocols. of 8 mice were immunized subcutaneously (S.C.) on day 1 with 250 μL of PBS, pH 7.4, containing 2.35 μg of total HA 1/3 (about specific HA from each strain) incorporated into PLGpS microparticles PLGpS or. microparticles formulated in the presence of BAY R1-005, DC-Chol or PCPP. Sera obtained on days +21, +42 and +57 and were evaluated for the presence of anti-Flu (trivalent) IgG antibodies (A/Texas, A/Johannesburg and B/Harbin) using an enzyme-linked immunosorbent assay (ELISA).

Figures 15a to c show the strain specific hemagglutination inhibition antibody assay (i.e. A/Texas (Figure 15a), A/Johannesburg (Figure 15b) and B/Harbin (Figure 15c)) responses (days +21, +42 and

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+57) following a single dose subcutaneous administration. Groups of 8 mice were immunized subcutaneously (S.C.) on day 1 with 250 μL of PBS, pH7.4, containing either 2.35 μg of total HA incorporated into PLGpS microparticles or 2.35 μg of total HA into PLGpS microparticles coencapsulating BAY R1-005, Chol or PCPP. Sera obtained on days +21, +42 or +57were evaluated for the inhibition of hemagglutination of erythrocytes.

10 Figure 16 shows the results of a protection study performed on mice immunized with a single dose of PLGpS/Flu (trivalent) microparticles or PLGpS/Flu (trivalent) coencapsulating BAY R1-005, DC-Chol or PCPP. The mice were challenged with homologous live virus and monitored for weight changes and survival over a 2 week interval.

Figure 17a shows the serum IgG antibody responses in mice immunized subcutaneously (S.C.) following various immunization protocols by the transferring binding protein from Moraxella catarrhallis (Tbp-2). Groups of 5 mice were immunized on days 1, 28 and 43 with 250 μ L of PBS pH 7.4, containing 0.3 μ g of tbp-2 incorporated into PLGpS microparticles, physically mixed PLGpS microparticles or formulated with Alum. Sera obtained on days +14, +27, +42 and +55 were evaluated for the presence of anti-tbp-2 IgG antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figure 17b shows the serum IgG antibody subtype response profile for pooled bleeds obtained on day 55 from the study conducted as described in Figure 17a.

Figure 18 shows the IgG serum antibody responses in mice immunized intranasally (I.N.) with the transferrin binding protein from Moraxella catarrhallis (Tbp-2). Groups of 5 mice were immunized on days 1, 28 and 43 with either 10 μ L or 50 μ L of PBS, pH 7.4, containing 6 μ g of Tbp-2 incorporated into

microparticles or physically mixed with PLGpS microparticles. Sera obtained on days +14, +27, +42 and +55 were evaluated for the presence of anti-Tbp-2 IgG antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figure 19 shows the serum IgG antibody responses in guinea pigs immunized parenterally following various immunization protocols by the transferrin binding protein from Moraxella catarrhallis (Tbp-2). Groups of 2 guinea pigs were immunized intramuscularly with 5 μg 10 of Tbp-2 formulated with 400 μL of CFA on day 1 followed subcutaneously with 5 μg of Tbp-2 formulated in 500 μL of IFA on days 14 and 28, 5 μg of Tbp-2 formulated with 500 μL of Alum on days 1, 14 and 28 or $5.0 \mu g$ of Tbp-2 incorporated into PLGpS microparticles 15 on days 1 and 28. Sera obtained on days +40 and +56 were evaluated for the presence of anti-tbp-2 antibodies using an enzyme-linked immunosorbent assay (ELISA).

20 Figure 20 shows the serum IgG antibody subtype responses in mice immunized subcutaneously (S.C.) or orally (I.G.) following various immunization protocols by the recombinant protein rUrease from Helicobacter Groups of 8 mice were immunized on days 1, 28 and 56 with 250 μL of PBS pH 7.4, containing 10.0 μg 25 (S.C.) or 40.0 μg (I.G.) of rUrease incorporated into PLGpS microparticles or 10.0 μg (S.C.) or 40.0 (I.G.) of rUrease formulated in the presence of DC-CT-X, PCPP or LTincorporated into microparticles. Sera was obtained on day +85 and were 30 evaluated for the presence of anti-rUrease antibodies using an enzyme-linked immunosorbent assay (ELISA).

Figures 21a to b show the results of a protection study for the mice described in Figure 20 one month after challenge on day 85. rUrease activity (for the

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mice immunized by subcutaneous or oral routes) was measured in 1/4 of a whole stomach (antrum+corpus) 24 hours after the mice were killed.

5 DETAILED DESCRIPTION OF THE INVENTION

The novel polymers of the present invention are biocompatible, degradable to benign metabolites which may be present in the body and may possess biologically active moieties, such as cell bioadhesion groups, macrophage stimulators, poly amino acids and polyethylene glycol coupled to the polymer via at least one spacer molecule selected from α -hydroxy acids and α -amino acids. As such, the novel polymers possess functionality.

Methods are also described for the synthesis of polymers having advantageous properties for processing into microparticles containing biologically active materials and for which chemical modification with biologically active targeting groups is possible.

In the preferred embodiments, the copolymers are produced by the polymerization of α -hydroxy acids with pseudo- α -amino acids and terpolymers produced by the polymerization of two α -hydroxy acids with pseudo- α -amino acids. The copolymer or terpolymer may then be derivatised with biologically active targeting ligands via the amino acid subunit by covalently coupling with the free amino group directly or subsequent to further derivatization with a suitable spacer ligand.

30 Amino Acid Monomer Synthesis

In general, an N-protected serine (or cysteine) is cyclized via a Mitsunobu reaction (ref. 5) to give a four membered lactone (or thiolactone).

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This transformation gives rise to an ester (or thioester) linkage. It is important to have protection on the amine portion of the amino acid precursor that compatible with the reaction conditions. Preferentially the carbobenzyloxy (CBZ or Z) group is 5 used although other suitable functionalities, such as benzyl (Bn), para-methoxybenzyl (MeOBn), benzyloxymethoxy (BOM), tert-butyloxycarbonyl (t-BOC) [9-fluorenylmethyl) oxy]carbonyl (FMOC) or may 10 employed.

The synthesis of the N-Z-L-Serine $\beta-Lactone$ monomer was based on a modified procedure from the literature (ref. 6).

Copolymerization Of α -Hydroxy Acid And Amino Acid Containing Monomers and Functionalization of Amino Acid Sidechains

Two methods are applicable for copolymerization of α -hydroxy acid monomers. Polymerization via polycondensation or from the melt (bulk polymerization) are possible alternatives.

Ιt has been long known that condensation polymerizations are problematic as relatively molecular weight materials often result with competing side commonly giving reactions rise to unwanted byproducts (refs. 7 and 8).

However ring opening polymerization (ROP) of the cyclic dimers of α -hydroxy acids, such as glycolide and lactide, from the bulk phase was shown to proceed readily in the presence of a variety of catalysts to give polymers of high molecular weights with stannous octoate being preferred (refs. 9 to 15).

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There are numerous methods for preparing poly(amino acids) (refs. 16, 17 and 18) or pseudopoly (amino acids) (refs. 6 and 19).

The noted biodegradable properties of poly- α -hydroxy acids (in particular those of 50:50 D,L-lactide and glycolide) and poly(amino acids) has resulted in increased efforts to develop methods for incorporating amino acids into the backbone of α -hydroxy acid polymers (refs. 20 to 25).

Advances have been made in producing copolyesteramides containing α -hydroxy acid sub-units, such as lactide or glycolide, and α -amino acid sub-units, such as glycine or lysine (refs. 22, 23 and 26).

The degradation rate of the biodegradable polymer and the release rates of encapsulated materials from homopolymers of glycolide, lactide or from copolymers of these materials has been shown to be strongly influenced by their molecular weight and structure, such as degree of crystallinity and relative hydrophobicity or hydrophilicity. Specifically, microspheres formulated from higher molecular weight polymers derived from α -hydroxy acids degrade over longer periods of time than lower molecular weight analogs. Similarly, highly crystalline materials erode at rates much slower than amorphous analogs. This is related to the accessibility of water hydrolytically unstable ester linkages (ref. 27).

It has been established that random amorphous copolymers composed of 50% D,L-lactide and 50% glycolide exhibit the most advanced degradation rates (refs. 2 and 3) with 50% by weight remaining after approximately 6 weeks, when immersed in PBS buffer (pH = 7.4).

The copolyesteramides described above are semicrystalline materials which may suffer from prolonged retention at the site of administration long after the encapsulated materials are fully released.

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Since it would be advantageous to have a polymer that has degraded at or near the point when encapsulated material has been fully released, developed methods for randomly incorporating equal amounts of D,L-lactide and glycolide into a terpolymer which also contained pseudo- α -amino acid sub-units. terpolymer of relatively moderate molecular weight was used to ensure the amorphous terpolymer would retain mechanical strength sufficient for processing into films and microparticles exhibit yet satisfactory polymer degradation and release rates for entrapped materials.

The N-protected-L-serine lactone contains an ester bond which may be polymerized via transesterification catalysts (ref. 6). Additionally it has been shown that six-membered ring lactones, such as lactide glycolide, can be copolymerized with four-membered ring propiolactones by use of insertion/coordination type catalysts/initiators (ref. 13). It was expected that efficient transesterification catalysts, such as those from Sn reagents, would be required relatively sufficient reactivity of all monomer units was to be achieved.

We used the copolymerization of glycolide, D,L-lactide and N-Z-L-serine lactone mediated by stannous octoate. Deprotection of the CBZ group of the copolymer or terpolymer can be achieved by various methods. Solid phase catalytic reduction or acid catalysis (ref. 27) are two possibilities (Figure 1).

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resultant copolymer or terpolymer can be The further elaborated with targeting moieties such as cell adhesion epitopes, poly ethylene glycol (PEG) ligands for circulation, macrophage stimulators and poly amino acid grafts as depicted in Figure 2. A spacer unit may be incorporated, for example, an α -hydroxy acid or a pseudo- α -amino acid unit, and may be derivatised with the appropriate targeting units. polymer so formed has a molecular weight of from about 5,000 to about 40,000 daltons.

Microparticle Formation

The term "microparticle" as used herein refers to any particulate carrier greater than 1 micron in size which is used for the delivery of biologically active materials. The term "microsphere" as used herein refers to a microparticle containing one or more active ingredients (e.g. antigens, adjuvants, plasmid DNA).

flow diagram illustrating the process microparticle formation as described herein is shown in In general, the copolymer (PLG, PLGpZS or solubilized solely PLGpS) is or with additional excipients present in a compatible solvent, such as dichloromethane, ethyl acetate, acetone or mixtures thereof. Excipients included in the formulation, such as sucrose, mannose, trehalose or gelatin, serve as cryoprotectants or lyoprotectants. Other materials possessing known adjuvancy, such as BAY R1-005 (BAY) (ref. 29) or tripalmitoyl cysteine (TPC) (ref. 30) or 3b[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) (ref. 31) may be included during formulation.

A 1% to 2% copolymer solution of total volume 12 $\,$ mL is preferably prepared. To this solution is added

800 μL of phosphate buffered saline (PBS) or 800 μL of antigen solution (concentration typically from 1 to 2 mg/mL) in PBS or other stabilizing buffers which may contain additional excipients. Other material 5 possessing known adjuvancy, such poly[di(carboxylatophenoxy) - phosphazene] sodium salt, (Virus Research Institute, Cambridge MA) (PCPP) cholera toxin or subunits thereof may be included during formulation. This mixture is then homogenized to form a water in oil emulsion. Once formed, this 10 mixture is dispersed into 100 mL of a 0.5% to 10.0% solution containing non-ionic emulsion stabilizers, such as poly vinyl alcohol (PVA), methyl cellulose or Triton X-100. This mixture is immediately homogenized to form a water in oil in water double 15 The average size and polydispersity of the emulsion. resultant droplets can be conveniently measured through use of the Coulter LS-100 light scattering detector. Typical size distributions when PBS or Antigen/PBS mixtures are encapsulated range from 1 to 20 microns 20 (with the majority less than 10 microns in size). solvent is then slowly removed via evaporation with gentle warming to harden the incipient microspheres. Once the solvent is removed the mixture is centrifuged 25 to collect the microspheres and repeatedly washed with deionized water to ensure complete removal of residual emulsion stabilizers. The microspheres are then frozen in a dry ice/acetone bath and lyophilized overnight to yield a white freely flowing powder of microspheres (typically 1.0 to 12 microns in size as determined by 30 light scattering measurements and directly verified via scanning electron micrography).

The particle generally comprise a polymeric matrix having a particle size of about 1 to about 50 μM which

comprises a polymer having the biologically-active material entrapped therein, with or without an adjuvant co-entrapped therein. The quantity of biologically-active material and adjuvant which may be incorporated into the polymeric matrix may vary widely and may comprise up to about 50 wt% of the total particle mass.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of medicine and in particular vaccination, diagnosis and treatment of infections with pathogens including bacteria and viruses. A further non-limiting discussion of suchuses is presented below.

15 Vaccine Preparation

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In an embodiment, immunogenic compositions, suitable to be used as, for example, vaccines, may be prepared from microparticles as disclosed herein. immunogenic composition containing biologically a · active immunogenic material can elicit an response by the host to which it has been administered including the production of antibodies by the host.

The immunogenic composition may be prepared as injectables, as liquid solutions or emulsions. The microparticles may be mixed with physiologically acceptable excipients which are compatible with the microparticles. These may include, water, saline, dextrose, glycerol, ethanol and combinations thereof. The vaccine may further contain additional substances such as wetting or emulsifying agents, pH buffering agents, or adjuvant*s* to further enhance effectiveness of the vaccines. Vaccines may administered parenterally, by injection subcutaneously or intramuscularly.

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Alternatively, the immunogenic compositions comprising microparticles formed according present invention may be delivered in a manner to elicit an immune response at mucosal surfaces. the immunogenic composition may be administered to mucosal surfaces by nasal, oral (intragastric), buccal rectal routes. Oral formulations may normally employed incipients, such as pharmaceutical grades of saccharin, cellulose and magnesium carbonate.

These compositions may take the form of solutions, suspensions. tablets, pills, capsules, sustained release formulations or powders. In order to protect microparticles and the encapsulated material contained within the core of the microparticle or which physically mixed with the microparticles, from gastric acidity when administered by the oral route, an acidic neutralizing preparation (such as a bicarbonate preparation) is advantageously administered concomitant with or directly after administration of the microparticles.

vaccines administered are in compatible with the dosage formulation, and in such amount as to be therapeutically effective, protective immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the subject's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune résponse. Precise amounts of microparticle and material having biological activity required to be administered depend on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms to milligrams. Suitable regimes for initial administration and booster doses

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also are variable, but may include an initial administration followed by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and thus vary from one host to another.

The polymers of the present invention as applied vaccine formulations, are also useful for vaccine strategies, such as with the use of DNA or antisense RNA. As microparticle carriers, the polymers of the present invention can be prepared to contain DNA 10 coding for a gene or genes for an antigenic portion of a virus such as the core protein or the envelope protein. As the DNA is released from the carrier, the host cells take up the foreign DNA, express the gene of interest and make the corresponding viral protein inside the cell. Advantageously, the viral protein enters the cells major histocompatibility complex pathway which evokes cell-mediated immunity. vaccine antigens in comparison, are taken up into cells and processed through the MHC class II system which stimulates antibody responses.

The DNA may be in the form of naked DNA which is free of all associated proteins and which does not . require a complex vector system. The desired gene may into a vector, such inserted as a encapsulated within the microparticle herein described, injected into the tissues of a mammal which expresses the gene product and evokes an immune response.

Antisense oligonucleotides can also be used in 30 conjunction with the polymers of the present invention. Nucleic acid sequences may be designed to bind to a specific corresponding mRNA sequence for protein to inhibit the production of the protein.

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microparticles described herein, can be used to deliver such antisense nucleotides (oligonucleotides or expressed nucleotides) as a therapeutic strategy for the treatment of various immunological diseases as well as hypertension, cardiovascular disease and cancer.

The slow-release characteristic of the polymer microparticles developed herein also has use in the field of pharmacology where the microparticles can be used to deliver pharmacological agents in a slow and continual manner. A wide range of drugs, such as antihypertensives, analgesics, steroids and antibiotics, can be used in accordance with the present invention to provide a slow release drug delivery system.

The polymer in the form of a film having a biological agent entrapped or physically admixed thereto, may also have use as a coating for surgical implants and devices. For example, the polymer as a film having antibiotic incorporated therein can be used to coat surgically implanted catheters in order to provide continual slow-release of antibiotics to combat infection.

The microparticle carrier may also be useful as a diagnostic agent. Together with the appropriate antibody, imaging agents can be incorporated with the microparticles. In this manner diseased tissues can be targeted and imaged in order to identify or monitor the clinical course of a disease.

The polymers, as microparticles, also have use in diagnostic kits when used in conjunction with appropriate antibodies.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can

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be obtained by reference to the following specific These Examples are described solely for Examples. purposes of illustration and are not intended to limit the scope of the invention. Changes in the form and substitution of equivalents are contemplated circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms intended in a descriptive sense and not purposes of limitations.

Methods of chemistry, organic chemistry, polymer chemistry, protein biochemistry and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1:

This Example illustrates the preparation of N-Z-L-Serine $\beta\text{-Lactone.}$

The preparation of this cyclic N-protected amino acid lactone was based on a modified procedure in which N-protected- α -amino acid is reacted to yield cyclized pseudo- α -amino acid monomer (ref. 6). glassware was pre-dried overnight in an oven set at 120°C. Prior to use it was cooled in a desiccator and purged under a stream of dry nitrogen for 10 minutes.

To a 1L three necked round bottomed flask under nitrogen was added triphenylphosphine (TPP; Aldrich; 7.87 mL; 50 mmol; FW: 174.16). To this was added 200 mL of anhydrous acetonitrile (CH₃CN; Aldrich): anhydrous tetrahydrofuran (THF; Aldrich) solution (volume ratio 85:15) via syringe and stirred until the solid TPP was dissolved. To this solution diethyl

azodicarboxylate (DEAD; Aldrich; 7.87 mL; 50 mmol; FW: 262.29) was added via syringe and the solution stirred at room temperature for 30 minutes. The solution was then cooled to about -45°C to -48°C by immersing the reaction vessel in an acetonitrile/dry ice bath. Once 5 the internal temperature of the solution reached about -45°C, a solution of N-Carbobenzyloxy-L-Serine (N-CBZ-L-Serine; Sigma; 11.94 g; 49.8 mmol; FW: 239.2) in 200 mL of anhydrous CH₃CN:THF (volume ratio 85:15) slowly added via dropping funnel over a period of 1 10 The temperature of the solution was maintained about -45°C during the addition and allowed to slowly warm to room temperature once the addition was complete with continuous stirring overnight. 15 reaction results in the formation of an ester bond between the serine hydroxyl side chain carboxylic acid in the presence of the CBZ protected $\alpha\text{--}$ Upon completion of the amino group. reaction the solvents were removed via evaporation (35°C to 45°C). This yields a yellow oil/slurry (\sim 35 g). 20 slurry was added 50 mL of dichloromethane:ethyl acetate (volume ratio 85:15) solution which results in the precipitation of 1,2-dicarbethoxyhydrazine byproduct. This material was removed by filtration under vacuum 25 followed by solvent removal via evaporation. The above procedure can be repeated to further remove residual byproducts. The waxy solid crude material was then purified via silica gel column chromatography with eluent 85:15 dichloromethane:ethyl acetate as solvent. The product serine lactone can be identified via thin 30 layer chromatography as this material has an $R_{\rm f}\mbox{ of }0.75$ on silica plates eluted with 85:15 dichloromethane:ethyl acetate when stained with a 1M H₂SO₄ solution and is also UV visible. The product was

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recrystallized from ethyl acetate:hexane (~1L), filtered and dried in vacuo.

A clean white solid is obtained in 40% yield after recrystallization with a melting point (Tm = 133-134°C) and all other physical parameters (NMR, IR, mass spectroscopy, elemental analysis) conforming to that previously demonstrated (ref. 6).

Example 2:

This Example illustrates the preparation of the copolymer poly-D, L-Lactide-co-Glycolide-co-pseudo-Z-Serine Ester (PLGpZS) as shown in Figure 1.

Glassware was pre-dried overnight. Prior to use it was cooled in a vacuum desiccator. Additionally the polymerization vessel (glass ampule) must siliconized (SurfaSil; Pierce; 2% solution in toluene) and all transfer reactions and additions of reagents and monomers to polymerization vessel must be conducted glove а box maintained under a dry nitrogen environment.

Prior to polymerization the D,L-lactide (2,6-dimethyl-1,4-dioxane-2,5-dione; Aldrich; FW: 144.13) and glycolide (Boehringer Ingelheim; FW: 116.096) was recrystallized from anhydrous ethyl acetate in the glove box and dried in vacuo for about 2 days. Once fully dried the monomers can be stored in the glove box with the freshly recrystallized serine lactone (stored at 0°C) of Example 1 brought directly into the glove box. All monomers and catalyst/initiators were weighed and transferred to glass ampules within the glove box.

The total combined mass of monomer transferred to the ampoule typically ranges from 1g to 5g with the molar ratio of D,L-lactide:glycolide:serine lactone ranging from 42.5:42.5:15.0 to 49.0:49.0:2.0. A molar

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ratio D, L-lactide:glycolide:serine lactone of of 47.5:47.5:5.0 was used in the preferred embodiment. A stock solution of catalyst (stannous 2-ethylhexanoate (Sn(Oct)₂; Sigma; FW 405.1, 1.25 g/mL) in anhydrous chloroform (Aldrich) was prepared in the glove box and added via microsyringe to the glass ampule (molar ratio of catalyst to monomer = 1/1000). A stir bar was also placed in the ampule. A greased ground glass joint valve was placed on the ampule to preserve the inert environment during removal from the glove box. The ampule was then directly placed on a vacuum line with slow removal of chloroform by evaporation. The ampules were then placed in an oil bath at ca. 120°C to bring all reagents into the melt followed by flame sealing and placement in a thermoregulated oven at 120°C for 28After reaction the ampules are quenched by placing in liquid nitrogen and stored at -20°C until further work up. The ampule was cracked and crude polymer recovered by dissolving in chloroform. solvent was removed by evaporation and crude polymer dried in vacuo to give an amber crystalline material (yield 80% to 90%).

polymer was purified by dissolving in chloroform. filtering off insoluble material and precipitating into hexane (Aldrich). The polymer was recovered by filtration and this procedure was repeated to ensure the complete removal of unreacted monomer. The polymer was dried in vacuo for about 2 days to give a clean white powder in 30% to 35% overall yield after second precipitation. The molecular weight of this material was dependent on reaction time with typical values of Mw =17,000-22,000, 6,500-8,000. Mn = Differential calorimetry scanning (DSC) analysis. indicates a single transition indicative of a random

amorphous polymer. Glass transitions (Tg) range from 39°C to 43°C dependent on the molecular weight of the material obtained. The serine content of the polymer was determined by amino acid analysis (AAA) diagnostic 5 the phenylthio isocyanate serine derivative obtained by hydrolysis of the polymer, (integration of aromatic residues of the CBZ protecting group on serine relative to the glycolide and D,Llactide sub-units) and elemental analysis (nitrogen present only in the side chain of serine). 10 The AAA analysis typically indicated 1.7% to 2.1% content with the 1H NMR analysis indicating 2.0% to 2.5% and the elemental analysis indicating 2.4% to 3.4% serine respectively. IR analysis of the polymer was diagnostic for the presence of ester, carbamate and 15 hydroxyl groups.

¹H NMR also allowed for the determination of the relative incorporation efficiencies of all monomer components under the stated reaction conditions. Typical ratios of D,L-lactide:glycolide:Z-serine found in purified polymer are reproducibly 52.0% to 54.0% D,L-lactide, 41.0% to 43.5% glycolide and 2.0% to 2.5% Z-serine respectively.

¹H NMR and ¹³C NMR signal intensities for resonances unique to glycolide or D,L-lactide are well resolved from each other and sensitive to sequence effects. From the observed patterns a random sequence distribution is supported.

30 Example 3:

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This Example illustrates the preparation of the copolymer poly-D,L-Lactide-co-Glycolide-co-pseudo-Serine Ester (PLGpS) as shown in Figure 1.

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All glassware was pre-dried overnight. Prior to use it was cooled in a vacuum desiccator and purged under a stream of dry nitrogen for 10 minutes. All reactions were conducted under inert atmosphere of dry nitrogen.

To a 2 necked 100 mL round bottomed flask equipped with a stir bar was placed 400 mg of polymer (PLGpZS). To this was added a 10 mL solution of 30% hydrogen bromide in acetic acid (Aldrich; FW: 80.92) which was sufficient for slurry formation. The slurry was stirred for 30 to 45 minutes and quenched by dropwise addition of anhydrous diethyl ether (Aldrich) followed anhydrous methanol (Aldrich). This results polymer precipitation which was then isolated by vacuum filtration. The crude polymer precipitate was washed with diethyl ether and reprecipitated from chloroform: hexane. The purified polymer was dried in vacuo for about 2 days to give a clean white powder in 50% to 60% overall yield. The molecular weight ranged from Mw = 15,000-18,000, Mn = 5,000-6,500. The rate of deprotection of the CBZ group is faster than competitive cleavage of the ester backbone HBr/Acetic acid. However, under these conditions there is broadening of the molecular weight distribution and reduction in the molecular weight of the product as a consequence of using this reagent. This trend can be reduced by conducting the reaction for shorter time intervals or eliminated by removing the protecting group via hydrogenation using hydrogen in the presence of palladium on charcoal. DSC analysis indicates a single transition indicative of a random amorphous polymer. Glass transitions (Tg) range from 42°C to 45°C depending on the molecular weight of the material The serine content of the polymer was obtained.

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determined by amino acid analysis (AAA) diagnostic for the phenylthio isocyanate serine derivative obtained by hydrolysis of the polymer and elemental analysis (nitrogen present only in the side chain of serine). The AAA analysis typically indicated 1.4% to 1.7% serine content and the elemental analysis indicating 2.0% to 2.7% serine respectively. IR analysis of the polymer was diagnostic for the presence of ester, amine

Example 4:

and hydroxyl groups.

This Example illustrates the production of a film from the copolymers synthesized in Examples 2 and 3.

To produce the film, 50 mg of poly-D,L-Lactide-co-Glycolide (PLG) (Mw = 31,000), poly-D,L-Lactide-co-Glycolide-co-pseudo-Z-Serine Ester (PLGpZS) (Mw poly-D, L-Lactide-co-Glycolide-co-pseudo-Serine Ester (PLGpS) (Mw = 19,000) was weighed out and placed in a 10 mL beaker. Anhydrous chloroform (1 mL) was added to dissolve the copolymer. This solution was filtered and added dropwise to a microscope slide placed in a petri dish. The petri dish was then covered with a 250 mL beaker to ensure slow evaporation over 48 hours. The resultant films were translucent contact angle measurements performed using goniometer gave average values of 75° for PLG, 75° for

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PLGpZS and 68.2° for PLGpS respectively. Thus the PLG and PLGpZS copolymers are of comparable hydrophobicity with the PLGpS copolymer proving to be slightly more hydrophilic and of higher surface energy.

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Example 5:

This Example illustrates the process of microparticle formation encapsulating PBS or antigen/PBS (microsphere formation).

A flow diagram illustrating the process of microparticle formation as described herein is shown in Figure 3.

Specifically, 100 mg of copolymer was added to 12 mL of dichloromethane. To this was added 800 uL of phosphate buffered saline (PBS) solution or 800 µL of non-proteolytic Hin-47 analog(concentration typically from 1 to 2 mg/mL) in PBS. This mixture was then homogenized (20 seconds at 6,000 rpm). Once formed this mixture was dispersed into 100 mL of a aqueous solution of poly vinyl alcohol (PVA) immediately homogenized (40 seconds at 8,000 rpm) to form a water in oil in water double emulsion. Typical size distributions when PBS or a typical antigen (Hin-47/PBS) is used as encapsulant are depicted in Figure Polydisperse microparticles (with the majority less than 10 microns in size) were formed under these The solvent was then slowly removed via conditions. evaporation and the microspheres collected centrifugation. The particles were washed (5X) with deionized water and then frozen in a dry ice/acetone bath and lyophilized overnight to yield a white freely flowing powder of microspheres (typically 1.5 to 10 microns in size as determined by light scattering measurements and directly verified via scanning

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micrography). electron representative Α scanning electron micrograph for PLGpZS or PLGpS microspheres encapsulating PBS is shown in Figure 5. representative scanning electron micrograph for PLGpZS or PLGpS microspheres encapsulating a typical antigen (non-proteolytic Hin-47 analog) in PBS is shown in Figure 6.

By the method stated above microparticles containing several different antigen(s) and/or antigen(s) + adjuvant have been prepared (see Tables 1 and 5).

Example 6:

This Example illustrates the microparticle core loading efficiency and antigen epitope recovery from such microparticles.

Two variations of the same method were employed to determine the antigen content or "core loading" of the microparticles isolated. Amino acid analysis performed on the hydrosylates of microparticles obtained by either acid hydrolysis (6M HCl) of the solid particles or by base/SDS hydrolysis (0.1N NaOH/1% SDS) followed by neutralization with 0.1N HCl. Alternatively, the solid microspheres can be dissolved in DMSO a compatible solvent solubilizing both polymer and protein and amino acid analysis performed directly on the lyophilized sample. The acid or base mediated hydrolysis proved to be the preferred method giving the most reproducible results (+/-5%). Where available a validated Enzyme Linked Immunosorbant Assay polyclonal assay was performed on the hydrosylates to determine the epitope equivalence.

Specifically, for the quantitation of non-proteolytic Hin-47 analog antigen by ELISA the non-

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proteolytic Hin-47 analog antigen was captured on affinity purified guinea pig anti-Hin47 coated microtitre wells (Add 50 μL of a 2 $\mu g/mL$ solution of non-proteolytic Hin-47 analog antigen per well), which have been blocked with 5% skim milk in PBS. The antigen present was detected by an affinity purified rabbit anti-Hin-47 followed by horse radish peroxidase F(abs)2, donkey anti-rabbit IgG. To develop the color of this reaction 100 μL of the substrate H_2O_2 (9 parts) in the presence of tetramethylbenzidine (TMB) (1 part), and the reaction progress terminated by addition of 50 μL of a 2M sulphuric acid solution to each well. intensity of the color (read at 450 nm) was directly proportional to the amount of non-proteolytic Hin-47 analog in the well. The concentration of nonproteolytic Hin-47 analog in each test sample was derived by extrapolation from a standard curve which was obtained by multiple dilutions of a reference sample. All samples were analyzed in duplicate.

The amino acid analysis for core loading and Hinspecific polyclonal ELISA analysis of epitope recovery for microparticles encapsulating nonproteolytic Hin-47 analog or non-proteolytic Hin-47 analog plus BAY R1-005 within PLG, PLGpZS and PLGpS copolymers is shown in Table 2. The core loadings for microparticles prepared solely from Hin-47 range from 20% to 43% with from 10% to 31% of the preserved during formulation. The core loadings for microparticles prepared from non-proteolytic Hin-47 analog in the presence of BAY R1-005 range from 26% to 59% (an absolute increase of 6% to 16%) with from 20% to 39% (an absolute increase of 4% to 18%) of the epitope preserved during formulation. Thus formulation in the presence of BAY R1-005 concomitantly increases

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the loading efficiency and serves to protect the epitope.

The amino acid analysis of core loading for microparticles encapsulating rD-15 within PLG, PLGpZS and PLGpS copolymers is shown in Table 3. Core loadings ranging from 39% to 44% were obtained. This protein was membrane derived and thus more hydrophobic than the non-proteolytic Hin-47 analog of the previous example. Increased encapsulation efficiencies relative to non-proteolytic Hin-47 analog of Example 10 were routinely observed when using this antigen.

The amino acid analysis for core loading epitope recovery for microparticles encapsulating a cocktail of non-proteolytic Hin-47 analog and rD-15 (1:1 cocktail) within PLG, PLGpZS and PLGpS copolymers is shown in Table 3. Ιt was expected coencapsulation of non-proteolytic Hin-47 analog in the presence of rD-15 would result in higher overall loading efficiencies. Core loadings ranging from 35% to 62% were obtained with from 8% to 26% of the nonproteolytic Hin-47 analog epitope preserved formulation. The overall loading efficiency of the hydrophilic component (non-proteolytic analog) was increased by coencapsulation with rD-15 when formulated with PLGpZS or PLGpS copolymers. effect was not observed when PLG was used. Irrespective of copolymer formulation similar non-proteolytic Hin-47 analog epitope recovery as compared with Example 10 was obtained.

The amino acid analysis of core loading for microparticles encapsulating Flu X-31 or Flu X-31 and BAY R1-005 within PLG, PLGpZS and PLGpS copolymers is shown in Table 4. Core loadings ranging from 26% to 40% were obtained with Flu X-31 and for Flu X-31 in the

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presence of BAY R1-005 core loadings ranging from 31% to 53% were obtained. Thus, an absolute increase of 5% to 13% was obtained when formulating in the presence of BAY R1-005. The only exception was the formulation of Flu X-31 with BAY R1-005 and PLGpS where an absolute decrease of 9% was observed.

A core loading of 21% for PLGpS microparticles encapsulating Flu A-Texas was obtained. For PLGpS microparticles encapsulating Flu A-Texas in the presence of BAY R1-005 a core loading of 32% was obtained.

When a different strain of Flu was employed (A-Texas) with BAY R1-005 and PLGpS copolymer the tendency to increase the encapsulation efficiency was once again observed. When formulating PLGpS with Flu A-Texas in the presence of BAY R1-005 an absolute increase of 11% was obtained. This suggests that the formulation for PLGpS and proteins in the presence of BAY R1-005 must be optimized in each case. As in Example 10, the addition of the coadjuvant BAY R1-005 has resulted in higher loading efficiencies.

The amino acid analysis of core loading for microparticles encapsulating Flu (trivalent) vaccine or Flu (trivalent) vaccine and BAY R1-005, DC-Chol or PCPP within PLGpS copolymers is shown in Table 6. Excellent core loading efficiencies ranging from 72% to 104% were obtained using the procedure of Example 5. The improvement in encapsulation efficiencies is a general result observed when the optimum concentration of protein is used to form the primary emulsion. In this case, the optimum concentration was experimentally determined to be about 0.5 mg/mL.

The amino acid analysis for core loading and rUrease specific polyclonal ELISA analysis of epitope

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recovery for microparticles encapsulating rUrease or rUrease plus DC-Chol, PCPP or CT-X within copolymers is shown in Table 7. Epitope recovery is defined as the difference between the total protein obtained by amino acid analysis and total protein obtained by polyclonal ELISA expressed as a percentage. The core loadings for microparticles prepared solely from rUrease is about 46% with about 72% of the epitope recovered during formulation. Similarly the core loadings microparticles prepared from rUrease in the presence of DC-Chol is about 44% with about 69% epitope recovered. Slightly higher total protein was determined microparticles prepared from rUrease in the presence of A core loading of 67% and epitope recovery of about 55% was found for this combination. rUrease/CT-X combination, the total core loading for rUrease could not be estimated by amino acid analysis since both rUrease and CT-X contribute to this value. A polyclonal ELISA assay for CT-X was developed and microparticle hydrosylates assayed for total rUrease and CT-X separately. An epitope recovery of 53% was found for rUrease and 59% for CT-X respectively. PAGE and Western blots performed on these microparticle hydrosylates confirmed the presence of non-degraded rUrease and CT-X.

Suitable controls were performed to ensure that the polyclonal ELISA assay for rUrease was unaffected by additives, such as DC-Chol and PCPP. In the case of PCPP, the assay was often problematic and variable. No similar problem was found when assaying rUrease in the presence of DC-Chol.

Example 7:

This Example illustrates the *in vitro* release rates and total cumulative % recovery of protein for a model antigen (non-proteolytic Hin-47 analog)

encapsulated within PLG (used as a control for comparison purposes) and synthesized copolymers PLGpZS and PLGpS.

The PLG formulated into microspheres containing antigen was determined to be of molecular weight; Mw = 5 26,000 with a 50:50 ratio of D,L-Lactide:Glycolide. This material is of similar constitution and molecular weight to that obtained for copolymers PLGpZS and PLGpS of Examples 2 and 3. In a typical experiment, 14 mg of microspheres (core loading of non-proteolytic Hin-47 10 analog for PLG = 2.8 μ g/mg, PLGpZS = 5.7 μ g/mg and PLGpS = 2.5 μ g/mg as determined by amino acid analysis of the microsphere hydrosylates) was placed in a 2 mL eppendorf tube to which was added 1.0 mL of PBS (pH = The tube was then placed in a thermoregulated 15 7.4). oven maintained at 37°C without agitation. At various time intervals the PBS solution was extracted and analyzed for total protein via amino acid analysis. After each extraction the PBS solution was replaced with continued sampling to a maximum of 90 days. 20 control experiments, the majority of the microspheres (>80% by weight) prepared from PLG or PLGpS copolymers consumed were by day 45. The degradation of microspheres formulated from PLGpZS copolymers was observed to be somewhat slower requiring 60 days to 25 erode to essentially the same extent.

Similar antigen release trends were observed with each group of microparticles wherein small amounts of protein were released over the first few days. This diminished to near undetectable limits up to day 14 whereafter the protein release rate steadily increased to a maximum at about day 30. Subsequent to this time, the release of protein fell to levels again approaching the limit of detection. The total % cumulative

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recovery of protein from these samples ranged from 40% to 65% relative to the respective core loadings of each group of microspheres.

Figure 7A illustrates the release rate at specific time points for each sample and Figure 7B shows the % cumulative release for each sample examined. evident that under these conditions the best recovery of protein from microspheres follows the PLGpS>PLG>PLGpZS although the core loading for the PLGpZS microparticles was approximately twice that of the PLG or PLGpS analogs and this may influence the release rate. In addition, this matrix has been shown to degrade at a slower rate and thus there may be residual material within the microparticles. for this can be seen in Figure 7B whereby a marginal increase in protein recovery was observed from day 60 to day 90 for PLGpZS microparticles. Over this same time interval, protein recovered from PLG or PLGpS microparticles was essentially non-existent.

In control experiments, supernatant solutions of PBS obtained from periodic extractions of PBS loaded PLG, PLGpZS or PLGpS microparticles incubated at 37°C were monitored for pH changes. It is known that the erosion process for PLGA matrices is accompanied by changes in the рН microenvironment within microparticle (ref. 36). This can have a serious on protein stability as На induced conformational changes can ensue as a consequence of these changes. An indication of the magnitude of these changes can be obtained by monitoring the pH of the surrounding medium. We have found that incorporation of small percentages of amino acid sub-units within the copolymer can retard this process. This may be of importance during the release phase for proteins

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sensitive to acidic pH. Specifically when incubating ~ 10 mg of microparticles in 1.2 mL of PBS buffer (pH = 7.4) it required approximately 16 days for supernatant extractions of PLG (Mw = 26,000 daltons) to fall below By analogy the pH of supernatant extractions pH 5.0. obtained from PLGpZS (Mw = 20,000 approximately 2.0% serine incorporated), or PLGpS (Mw =18,000 daltons, approximately 1.7% serine incorporated) was determined to be approximately 5.5 respectively. The degradation rates for PLG and PLGpS examined in this study were similar (as measured by mass loss of matrix) whereas the PLGpS matrix degraded at a reduced rate.

Thus the *in vitro* release study demonstrates that a single dose delayed release delivery system can be achieved through use of polymeric microparticles formulated from PLGpZS or PLGpS copolymers. In addition as pH changes with matrix erosion can have a deleterious effect on the protein stability it may be advantageous to employ matrices derived from pseudoserine ester such as PLGpZS or PLGpS wherein there exists some buffering capacity for these changes during the protein release phase.

25 Example 8:

This Example illustrates the immunogenicity of non-proteolytic Hin-47 analog encapsulated or physically mixed with microparticles in mice which were immunized subcutaneously.

To examine the immunogenicity of non-proteolytic Hin-47 analog entrapped in PLG, PLGpZS and PLGpS microparticles formed in accordance with the present invention, groups of five, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories,

Wilmington, MA) were immunized subcutaneously (S.C.) with the following amounts of antigen in 250 μ L of PBS (pH 7.4) on days 1 and 35: PLG, PLGpZS and PLGpS microparticles prepared as described in Example 5 containing 0.2 μ g or 0.6 μ g of non-proteolytic Hin-47 analog (Figure 8A); and PLG microparticles prepared as described in Example 5 physically mixed with 0.8 μ g or 2.5 μ g of non-proteolytic Hin-47 analog (Figure 8B).

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained 10 encapsulated non-proteolytic Hin-47 analog or microparticles that were physically mixed with nonproteolytic Hin-47 analog. Sera were obtained on days +10, +24, +35, +45 and +60 and were evaluated for the presence of anti-Hin-47 IgG antibodies by antigen 15 specific ELISA. All samples were analyzed duplicate. Microtiter plate wells were incubated overnight at room temperature with 100 µL of 0.2 µg/mL non-proteolytic Hin-47 analog in 0.05M carbonatebicarbonate buffer (pH 9.0). 20 The plates were washed with PBS + 0.05% Tween 20 (operationally defined as washing buffer). Wells were incubated with 200 μL of 5% milk (operationally defined as blocking buffer). After washing with PBS + 0.05% Tween 20, the plates were incubated for 1 h at 37°C with 100 μL of 25 sample serially diluted in blocking buffer. Wells were washed with PBS + 0.05% Tween 20 and 100 μL of HRPconjugated antibody (goat anti-mouse IqG (H (Jackson), sheep anti-mouse IgG1 (Serotec), goat antimouse IgG2a (Caltag) or goat anti-mouse IgG2b (Caltag) 30 in blocking buffer was added to each well. After 1 hour incubation at 37°C, the wells were washed five times with PBS + 0.05% Tween 20 and 100 μL of freshly prepared colorizing substrate $[H_2O_2\ (9\ parts)\ and\ TMB$

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(1 part)] is added to each well. After 5 minutes incubation in the dark at room temperature the reaction is stopped by adding 50 μ L of a 2M H_2SO_4 solution and the optical density of the fluid in each well was determined at 450 nm using a microplate reader. A normal mouse sera pool was used to establish baseline optical density values in the assay. Hyperimmune mouse Hin-47 antiserum was used as a positive control. Pre-immune sera was used as negative control.

For serum IgA analysis the above procedure was conducted with the following modification. Microtiter plate wells were incubated overnight at room temperature with 100 μ L of 1.3 μ g/mL non-proteolytic Hin-47 analog in 0.05M carbonate-bicarbonate buffer (pH = 9.0), and 100 μ L of HRP conjugated rabbit anti-mouse IgA (ZYMED, CA) at 0.06 μ g/mL was added to each well.

For secretory IgA analysis, the above procedure was conducted with the following modification. 100 μL of HRP conjugated rat anti-mouse IgA (Biotin-Pharmigen) at 0.06 $\mu g/mL$ was added to each well.

The serum antibody titres following immunization are shown in Figures 8A and 8B. The results of immunizations indicate that antigen presented to the immune system entrapped in PLG, PLGpZS or microparticles (Figure 8A) was substantially immunogenic than soluble antigen at doses sub-optimal to that required with soluble antigen alone. addition, dose no dependence was observed with encapsulated non-proteolytic Hin-47 analog of dose 0.2 μg or 0.6 μg , whereas a marginal increase in titre was found with soluble non-proteolytic Hin-47 analog of dose 0.8 µg or 2.5 µg respectively.

The results of immunizations indicate that antigen presented to the immune system when physically mixed

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with PLG microparticles (Figure 8B) is marginally more immunogenic than soluble antigen at similar dose to that given with soluble antigen alone (0.8 μg or 2.5 μg). However, administration of antigen in soluble form or admixed with microparticles elicits a response several orders of magnitude less than that seen for antigen encapsulated within the microparticles demonstrating the advantages of encapsulation over soluble or physically mixing alone.

Interestingly, the IgG subtype profile (Figure 8C) for pooled serum obtained from the bleeds on day 35 and 60 for microparticle encapsulated non-proteolytic Hin-47 analog, microparticles physically mixed with nonproteolytic Hin-47 analog or soluble non-proteolytic Hin-47 analog shows that by day 35 IgG1 was the dominant subtype with some IgG2b detected irrespective of formulation. By day 60 it was evident that the IgG induced by non-proteolytic Hin-47 subtypes encapsulated within microparticles had undergone class switching such that IgG1, IgG2a and IgG2b are more equally represented. The IgG subtypes induced by nonproteolytic Hin-47 analog physically mixed particles or in soluble form by day 60 was nominally the same as that determined for day 35 with the IgG1 subtype dominant.

Thus, it can be concluded that the qualitative nature of the immune response mediated by microparticles encapsulating antigen is substantially different than that obtained by physically mixing with microparticles or by soluble antigen alone. The presence of IgG2a subtype in the BALB/c mouse model is generally accepted to be indicative of a TH_1 pathway, and IgG1 indicative of a TH_2 pathway. It is evident that via the subcutaneous route the TH_2 pathway is

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favored for soluble antigen or for antigen physically mixed with microparticles, whereas with antigen encapsulated within microparticles a more balanced $\mathrm{TH}_1/\mathrm{TH}_2$ response is attainable.

5 Example 9:

This Example illustrates the immunogenicity of non-proteolytic Hin-47 analog entrapped in PLG, PLGpZS and PLGpS microparticles formed in accordance with the present invention, in mice immunized intragastrically.

Groups of five, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized intragastrically (I.G.) with the following amounts of antigen in 250 μ L of 0.15 M NaHCO₃ (pH 9.0) on days 1, 7, 14 and 57: PLG, PLGpZS and PLGpS microparticles prepared as described in Example 5 containing 4.0 μ g of non-proteolytic Hin-47 analog; and PLGp, PLGpZS and PLGpS microparticles prepared as described in Example 5 physically mixed with 4.0 μ g non-proteolytic Hin-47 analog (Figure 9A).

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained encapsulated non-proteolytic Hin-47 analog or microparticles that were physically mixed with non-proteolytic Hin-47 analog. Sera were obtained on days +13, +35, +56 and +78 and were evaluated for the presence of anti-Hin-47 IgG and IgA antibodies by antigen specific ELISA as described in Example 8.

Sera and intestinal washes were examined for the presence of Hin-47-specific antibodies. To detect and quantify anti-Hin-47 IgG and sIgA in the intestinal lumen, mice were sacrificed by cervical dislocation, their small intestines removed and examined for the presence of antigen-specific antibodies. Individual small intestines were detached from the pyloric

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sphincter to the caecum and everted over capillary The everted intestines were incubated in $5~\mathrm{mL}$ of ice cold enzyme inhibitor solution (0.15 M NaCl, 0.01 M Na₂HPO₄, 0.005 M EDTA, 0.002 M PMSF, 0.05 U/mL Aprotinin, and 0.02% v/v NaN3) for 4 hours. Intestines removed and the supernatants clarified centrifugation (1000 xg, 20 minutes) and stored at 0°C until assayed. Anti-Hin-47 IgG and sIgA titres samples were determined by Hin-47-specific ELISA as described above but a goat anti-mouse IgA antiserum was used in place of the goat anti-mouse IgG antiserum.

The serum IgG Hin-47-specific antibody titres following I.G. immunization is shown in Figure 9A. These results indicate that an antigen (non-proteolytic Hin-47 analog) incorporated into PLG, PLGpZS or PLGpS microparticles was substantially more immunogenic than soluble antigen of similar dose (4 µg) and better than antigen that was physically mixed with microparticles when delivered by the intragastric route. experimentally determined that a dose of antigen (20 μ g) which was five times that which was administered encapsulated within microparticles (4 µg) was required to elicit an essentially equivalent response. This result is atypical for most proteins as there are many examples where in excess of 100 μg of antigen is required to elicit any significant serum IgG antibody response via the intragastric route.

The IgG subtype profile (Figure 9B) for pooled serum obtained from the bleeds on day 56 and 78 for microparticle encapsulated non-proteolytic Hin-47 analog, microparticles physically mixed with non-proteolytic Hin-47 analog or soluble non-proteolytic Hin-47 analog shows a similar trend as that observed in Example 8. The IgG1 subtype was dominant when antigen

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was delivered in soluble form or when physically mixed with microparticles. Non-proteolytic Hin-47 analog encapsulated within microparticles exhibits a more balanced profile with IgG1 and IgG2a more equally represented. Thus via the intragastric route with antigen encapsulated within microparticles a more balanced TH_1/TH_2 response was attainable.

Figure 9C shows the results for anti-Hin-47 IgA antibody responses obtained from bleeds on day 78. With soluble antigen at 4 μg per dose no detectable serum IgA was found, however at 20 µg per dose responders were observed. A single significant response observed with antigen encapsulated within microparticles and moderate responses observed for antigen encapsulated within PLGpZS or PLGpS microparticles. Similarly modest response was seen for antigen physically mixed with PLG, PLGpZS or PLGpS microparticles. The highest average levels of serum IgA were obtained for antigen encapsulated within PLGpS microparticles.

The intestinal lavage conducted on day 78 revealed minimal levels of IgG or sIgA specific for Hin-47 analog in the mucosal secretions obtained from nonproteolytic Hin-47 analog encapsulated within PLGpZS or PLGpS microparticles. This is likely due to verv low levels of encapsulated administered in this experiment (4 µg per dose) as oral doses of antigen ranging from 30 μg to 100 μg are usually required to elicit a significant mucosal response in the absence of any other mucosal adjuvants.

Example 10:

This Example illustrates the immunogenicity of non-proteolytic Hin-47 analog entrapped in PLG, PLGpZS

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and PLGpS microparticles formed in accordance with the present invention, in mice immunized intranasally.

Groups of five, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized intranasally (I.N.) with the following amounts of antigen in 25 μ L of PBS (pH 7.4) on days 1, 7, 14 and 57: PLG, PLGpZS and PLGpS microparticles prepared as described in Example 5 containing 4.0 μ g of non-proteolytic Hin-47 analog; and PLG, PLGpZS and PLGpS microparticles prepared as described in Example 5 physically mixed with 4.0 μ g non-proteolytic Hin-47 analog (Figure 10A).

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained encapsulated non-proteolytic Hin-47 analog or microparticles that were physically mixed with non-proteolytic Hin-47 analog. Sera were obtained on days +13, +35, +56 and +78 and were evaluated for the presence of anti-Hin-47 IgG and IgA antibodies by antigen specific ELISA as described in Example 8.

Sera and lung lavage washes were examined for the presence of Hin-47-specific antibodies. To detect and quantify anti-Hin-47 IgG and sIgA in the respiratory tract, mice were sacrificed by cervical dislocation, a small incision was made in the trachea and a tube inserted through the trachea into the lungs where 400 μL of an enzyme inhibitor solution (0.15 M NaCl, 0.01 M Na₂HPO₄, 0.005 Μ EDTA, 0.002 М PMSF, 0.05 Aprotinin, and 0.02% v/v NaN3) was injected. solution was then withdrawn, placed on ice and the supernatants clarified by centrifugation (1000 xq, 20 minutes) and stored at 0°C until assayed. Anti-Hin-47 IgG and sIgA titres in samples were determined by Hin-47-specific ELISA as described in Example 8.

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The serum IgG Hin-47-specific antibody titres following I.N. immunization is shown in Figure 10A. These results indicate that an antigen (non-proteolytic Hin-47 analog) incorporated into or physically mixed with PLG, PLGpZS or PLGpS microparticles was more immunogenic than soluble antigen of similar dose (4 μg). Essentially equivalent response was obtained for antigen that was encapsulated or physically mixed with microparticles by the intranasal route.

The IgG subtype profile (Figure 10B) for pooled 10 serum obtained from the bleeds on day 56 and 78 for microparticle encapsulated non-proteolytic Hin-47 analog, microparticles physically mixed with proteolytic Hin-47 analog or soluble non-proteolytic 15 Hin-47 analog illustrated a trend different from that shown in Example 8 or Example 9. Non-proteolytic Hin-47 analog encapsulated within microparticles exhibited a more balanced profile with IgG1, IgG2a essentially equally represented at either timepoint 20 (day 56 or day 78). For antigen delivered in soluble form or when physically mixed with microparticles, a similar profile was seen by day 78 where significant levels of IgG2a can also be seen.

Thus, via the intranasal route, the TH_1/TH_2 response was essentially the same irrespective of whether antigen was encapsulated within microparticles, physically mixed with microparticles or in soluble form.

Figure 10C shows the results for anti-Hin-47 IgA antibody responses obtained from bleeds on day 78. With soluble antigen no detectable serum IgA was found. However, significant response was seen for antigen that is encapsulated or physically mixed with microparticles. The highest levels obtained for antigen

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either encapsulated or physically mixed with PLGpS microparticles.

lung lavage conducted on day 78 revealed substantial differences with respect to IgG and sIgA detected from secretions. The anti-Hin-47 IgG antibody response (Figure from soluble 10D) antigen however negligible, antigen encapsulated microparticles or physically mixed with PLG · microparticles elicited significant levels of IgG from half of each group examined. The most reproducible response for IgG was found with antigen physically mixed with microparticles derived from PLGpZS or PLGpS where most or all mice examined possessed significant levels of IqG.

The anti-Hin-47 sIgA antibody response (Figure 10E) was similar to that obtained for IgG in Figure 10D. Soluble antigen response was negligible with antigen encapsulated within microparticles or physically mixed with PLG microparticles showing some response. The most significant response for sIgA was found with antigen physically mixed with microparticles derived from PLGpZS or PLGpS where most mice have reasonable levels of sIgA.

induction of local protection at mucosal surfaces is often associated with the presence IgG and sIgA in local secretions. Whereas it cannot be ruled the intranasal out that immunization has not also resulted in immunization of the upper respiratory tract, it is clear that for serum IgG antibody response encapsulated or physically mixed antigen with microparticles is effective. To induce local secretions of IgG and sIgA physically mixing antigen microparticles and more specifically microparticles formulated from either PLGpZS or PLGpS

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appears to be the more suitable method under these conditions.

Example 11:

This Example illustrates the immunogenicity of Flu-X-31 or Flu X-31 plus a co-adjuvant BAY R1-005 entrapped in PLG, PLGpZS and PLGpS microparticles formed in accordance with the present invention, in mice immunized subcutaneously.

Groups of six, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized subcutaneously (S.C.) with the following amounts of antigen in 250 μL of PBS (pH 7.4) on day 1: PLG, PLGpZS and PLGpS microparticles prepared as described in Example 5 containing 5.0 μg of Flu X-31 (1.5 μg HA) or 5.0 μg of Flu X-31 (1.5 μg HA) and 50 μg BAY R1-005 when administered in soluble form or approximately 20 μg BAY R1-005 when co-encapsulated (Figure 11).

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained encapsulated Flu X-31 or Flu X-31 with BAY R1-005. Sera were obtained on days +21 and +33 and were evaluated for the presence of anti-Flu X-31 IgG antibodies by antigen specific ELISA. All samples were analyzed in duplicate.

Microtiter plate wells were incubated overnight at room temperature with 500 μL of 4.0 $\mu g/mL$ Flu X-31 in 0.05M carbonate-bicarbonate buffer (pH = 9.6). The plates were washed with PBS + 0.05% Tween 20 (operationally defined as washing buffer). Wells were incubated with 200 μL of 5% skim milk (operationally defined as blocking buffer). After washing with PBS + 0.05% Tween 20, the plates were incubated for 1 h at

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37°C with 100 μ L of sample serially diluted in blocking Wells were washed with PBS + 0.05% Tween 20 buffer. and 100 μL of HRP-conjugated antibody (goat anti-mouse IgG (H + L), IgG1, IgG2a or IgG2b) in blocking buffer was added to each well. After 1 hour incubation at 37°C, the wells were washed 5X with PBS + 0.05% Tween 20 and 100 μL of freshly prepared colorizing substrate $[H_2O_2$ (9 parts) and TMB (1 part)] is added to each well. After 5 minutes incubation in the dark at room temperature the reaction is stopped by adding 50 μL of a 2M H₂SO₄ solution and the optical density of the fluid in each well was determined at 450 nm using a microplate reader. A normal mouse sera pool was used to establish baseline optical density values in the assay. Hyperimmune mouse Flu X-31 antiserum was used as a positive control. Pre-immune sera is used as negative control.

The serum antibody titres following immunizations are shown in Figure 11. The results of a single immunization (day 1) indicated that antigen presented to the immune system entrapped in PLG, PLGpZS or PLGpS microparticles was more immunogenic than soluble antigen alone. The most relevant results were obtained with Flu X-31 or Flu X-31 plus BAY R1-005 encapsulated within PLGpZS microparticles. Although a sub-optimal of BAY R1-005 was encapsulated within microparticles examined, the immunogenic response with PLGpZS microparticles also proved significantly higher than that obtained with soluble Flu X-31 and BAY R1-005 alone.

The studies presented in this Example demonstrate that viral antigens from influenza virus can be made more immunogenic and elicit high levels of serum IgG antibodies, when the antigens are entrapped in

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microparticles formed in accordance with the present invention. In addition the significantly higher immunogenicity displayed by the microparticle systems after a single immunization demonstrates the potential of these materials for development as single dosage vaccines.

Example 12:

This Example illustrates the immunogenicity of Flu-X-31 or Flu X-31 plus a co-adjuvant BAY R1-005 entrapped in PLG, PLGpZS and PLGpS microparticles formed in accordance with the present invention, in mice immunized intranasally.

Groups of six, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized intranasally (I.N.) with the following amounts of antigen in 25 µL of PBS (pH 7.4) on days 1 and 34: PLG, PLGpZS and PLGpS microparticles prepared as described in Example 5 containing 5.0 µg of Flu X-31 (1.5 µg HA) or 5.0 µg of Flu X-31 (1.5 µg HA) and 50 µg BAY R1-005 when administered in soluble form or approximately 20 µg BAY R1-005 when co-encapsulated (Figure 12).

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained encapsulated Flu X-31 or Flu X-31 with BAY R1-005. Sera were obtained on days +21, +33 and +92 and were evaluated for the presence of anti-Flu X-31 IgG antibodies by antigen specific ELISA as described in Example 10. All samples were analyzed in duplicate.

Mice immunized I.N. with soluble antigen, soluble antigen plus co-adjuvant or encapsulated antigen showed a similar anti-Flu X-31 IgG antibody response. Interestingly, a reduced (likely due to delayed

release) immunogenic response for encapsulated antigen plus co-adjuvant relative to soluble antigen, antigen plus adjuvant or encapsulated antigen on day +21 and +33 was shown when administered via the nasal route. However, after the second immunization (day 34) a significant boost in response with these groups was observed resulting in essentially similar immunogenicity on day +92 for all groups irrespective of adjuvant used.

The results of the I.N. immunizations described in this Example shows that the immunogenicity of an antigen or antigen plus a co-adjuvant cannot be significantly enhanced by entrapment in microparticles formed in accordance with the present invention.

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Example 13:

This Example illustrates the immunogenicity of Flu A-Texas or Flu A-Texas + BAY R1-005 encapsulated or physically mixed with microparticles in mice immunized subcutaneously.

It is known that most non-replicating viral vaccines require multiple doses for sufficient serum antibody titres to be protective. Thus, it is strongly desirable to achieve this by administering a single dose of antigen.

We sought to examine the immunogenicity of Flu A-Texas or Flu A-Texas plus a co-adjuvant BAY R1-005 which was entrapped in PLGpS microparticles or physically mixed with microparticles administered as a single dose, formed in accordance with the present invention. Groups of six, 6 to 8 week old female DBA2 mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized subcutaneously (S.C.) with the following amounts of antigen in 250 µL of PBS (pH 7.4)

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on day 1: PLGpS prepared as described in Example 5 containing 1.0 μg of Flu A-Texas (0.35 μg HA) or 10.0 μg of Flu A-Texas (3.5 μg HA) or 1.0 μg of Flu A-Texas (0.35 μg HA) and approximately 2.0 μg of BAY R1-005 or 10.0 μg of Flu A-Texas (3.5 μg HA) and approximately 20 μg of BAY R1-005 or microparticles prepared as described in Example 5 physically mixed with 1.0 μg of Flu A-Texas (0.35 μg HA) and 20 μg of BAY R1-005 or 10.0 μg of Flu A-Texas (3.5 μg HA) and 20 μg of BAY R1-005 or 10.0 μg of Flu A-Texas (3.5 μg HA) and 20 μg of BAY R1-005 (Figure 13).

The core loading of PLGpS microparticles containing Flu A-Texas and BAY R1-005 was determined amino acid analysis (Table 1). The mass microparticles administered was adjusted such that the required dose of Flu A-Texas (1.0 µg of Flu A-Texas (0.35 μg HA) for the low dose groups or 10.0 μg of Flu A-Texas (3.5 µg HA) for the high dose groups) delivered. Thus the dose of BAY R1-005 delivered for the high dose groups was ten fold greater than that of the low dose groups examined. It is expected that formulation conditions can be adjusted such that the quantity of BAY R1-005 co-encapsulated is comparable.

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained encapsulated Flu A-Texas or Flu A-Texas with BAY R1-005. Sera were obtained on days +21 and +42 or +57 and were evaluated for the presence of functional antibody via the hemagglutination inhibition antibody assay (HAI). All samples were analyzed in duplicate.

The influenza hemagglutination inhibition antibody assay was performed with heat-inactivated mouse serum that had been incubated for 30 minutes with 10% chicken red blood cells to remove non-specific inhibitors. Twofold dilutions of sera were added to a 96 well

microtiter plate and 8 HA units of virus suspension in an equal volume were added to each well and incubated at room temperature for 30 minutes. A 1.0% suspension of chicken red blood cells were added to each well and incubated at room temperature for 30 minutes. Positive and negative reference sera were included in the test to ascertain specificity and sensitivity of the test. Positive sera from influenza virus immunized animals. Negative sera from PBS immunized animals, titre should be less than or equal to 15. The HAI titres are expressed as the reciprocal of the highest dilution that completely inhibits hemagglutination of erythrocytes.

The results of a single immunization (day 1) Figure 13 indicate that by day +42 antigen presented to the immune system in soluble form or entrapped in PLGpS microparticles (Figure 13) elicits negligible or low functional antibody. For Flu (10 μg) presented to the immune system as a physical mixture with BAY R1-005 the HAI response elicited was 8x higher than that obtained with soluble antigen of similar dose and 6x higher that that obtained by PLGpS/Flu microparticles of either dose. relevant most results were obtained for PLGpS/Flu(A-Texas)/BAY R1-005 formulated microparticles wherein the HAI titre of the low dose group (1 μ g) was 8x that of soluble antigen and the high dose group (10 μg) was 32x that of soluble antigen. A moderate increase in the HAI titre was found for the additional control groups examining physical Flu with PLGpS microparticles or mixtures of mixtures of Flu, PLGpS microparticles and BAY R1-005. HAI responses elicited with physically mixed Flu and PLGpS microparticles was found to be 4x higher for the low

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dose group and 8x higher for the high dose group than that obtained with soluble antigen of similar dose. The HAI responses elicited for physical mixtures of Flu with PLGpS microparticles and BAY R1-005 was found to be 4x higher than that obtained with soluble antigen, irrespective of dose.

The studies presented in this Example demonstrate that viral antigens from influenza virus elicit high levels of functional antibodies when the antigens are entrapped in microparticles in the presence of an additional adjuvant (as determined by HAI titres), formed in accordance with the present invention. There is a dose dependence observed and in addition the significantly higher functional antibody (a correlate for protection) displayed by the microparticle antigen and adjuvant co-encapsulated systems after a single immunization further demonstrate the potential of these materials for development as single dosage forms.

20 Example 14:

This Example examines the effects of formulation conditions typically employed for microencapsulation on individual components of Flu (trivalent) vaccine.

Flu (trivalent) vaccine contains three homologous HA strains (A/Texas, A/Johannesburg and B/Harbin) in approximately equal amounts. The quantitation of each specific HA component has been determined by single radial diffusion assay (SRID) (ref. 32). assay employs polyclonal sera to detect HA component and is an effective indicator for conformational changes. The minimal concentration detectable with this assay for each component approximately 10 μ g/mL. A series of samples containing 2.0 mL of Flu (trivalent) vaccine (concentration = 265

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 μ g/mL) were prepared. For each of these samples, the concentration of A/Texas specific HA = 20.25 μ g/mL, A/Johannesburg specific HA = 20.60 μ g/mL and B/Harbin specific HA = 21.42 μ g/mL, respectively, was determined by SRID.

The effects of solvents typically employed in microencapsulation procedures, such as Dichloromethane (DCM) or Ethyl Acetate (EtOAc), were evaluated under conditions that simulate the homogenization procedure. Short sonication times (30 seconds) were used to model this. Additionally, small amounts of additives, such as BAY (a glyco-lipo-peptide) and DC-Chol (a cationic lipid), were examined to determine if improved recovery of antigen can be confirmed.

The scale of the reaction was designed to mimic the procedures as described in Example 5. The organic solvent was evaporated from each mixture prior to SRID analysis and suitable controls were examined to determine if the organic solvent, homogenizing method or addition of BAY or DC-Chol perturbed the results in any way.

The results of this study are shown in Table 8. The effects of sonication on the sample were minimal as shown by comparing entries 1 and 2. Entries 3 and 4 show that the recovery of antigen was affected by sonication in organic solvents like EtOAc or DCM. When EtOAc is employed, 75% of A/Texas specific HA and 78% A/Johannesburg specific HA is recovered. B/Harbin specific HA component was detectable by this method after treatment with EtOAc. This indicates that less than 50% of this component was actually recovered, as the lower detection limit is about 10 μ g/mL. When DCM was employed as solvent, 85% of A/Texas specific HA, 96% of A/Johannesburg specific

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HA and less than 50% of B/Harbin specific HA component were recovered respectively.

Entries 5 and 6 examine the influence of BAY or DC-Chol as additive in the organic phase with EtOAc as For this combination, all components were detectable (at levels > 50%) with 65% (BAY) or 82% (DC-Chol) A/Texas specific HA recovered, 82% (BAY) or 70% (DC-Chol) B/Harbin specific HA recovered and 87% (BAY) or 88% (DC-Chol) A/Johannesburg specific HA recovered respectively. Entries 7 and 8 examine BAY or DC-Chol as an additive in the organic phase with DCM as The assay indicates that some components were not completely recovered or that the assay is effected in some way. Specifically, 91% (BAY) or 92% (DC-Chol) A/Texas specific HA is recovered. Less than B/Harbin specific HA was recovered in either case. results for A/Johannesburg specific HA are inconclusive deviations from linearity with combinations. This occurred only when DCM was used as solvent.

Maximum recovery of all components was obtained for formulations employing Flu (trivalent) vaccine in EtOAc with BAY or DC-Chol as additive. This Example indicates that materials with lipophilic properties, such as BAY or DC-Chol, can be used to stabilize vaccine formulations containing sensitive components. Materials with these properties can function interfaces such that proteins are protected from the air/water hydrophobic surface during homogenization. Protein conformation is particularly sensitive to these effects. The SRID assay results reported in this Example support these conclusions.

Example 15:

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This Example illustrates the single dose immunogenicity of Flu (trivalent) or Flu (trivalent) + adjuvant cocktails coencapsulated within PLGpS microparticles in mice immunized subcutaneously.

Flu (trivalent) vaccine contains three homologous HA strains (A/Texas, A/Johannesburg and B/Harbin) in approximately equal amounts. The total HA content in 10.0 μ g of flu (trivalent) vaccine is 2.35 μ g. Each specific HA component has been determined by single radial diffusion assay (SRID) (ref. 32) to be 0.76 μ g A/Texas, 0.81 μ g A/Johannesburg and 0.78 μ g B/Harbin respectively.

In this study the dose of HA administered for each component strain was significantly less than that used in Example 13. For comparison, the monovalent vaccine used in Example 13 contained approximately 3.25 μg of A/Texas specific HA.

Mice were immunized by the subcutaneous route in the presence of adjuvants chosen based on Th_2/Th_1 profile; DC-Chol (a cationic lipid) and BAY R1-005 (glyco-lipo-peptide with more balanced Th_2/Th_1), PCPP (poly[di(carboxylatophenoxy) - phosphazene] sodium salt, (Virus Research Institute, Cambridge MA), primarily Th_2 . PLGpS microparticles have been shown to induce a more balanced Th_2/Th_1 profile, as described in Example 8.

Groups of eight 6 to 8 week old female DBA-2 mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized subcutaneously (S.C.) with the following amounts of antigen in 250 μ L of PBS (pH 7.4) on day 1: PLGpS microparticles prepared as described in Example 5 containing 10.0 μ g of Flu (trivalent - 2.35 μ g HA) or

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10.0 μ g of Flu (trivalent - 2.35 μ g HA) and BAY R1-005, DC-Chol or PCPP (Table 5).

The core loading of PLGpS microparticles containing Flu (trivalent) was determined via amino acid analysis (Table 6). Excellent recovery of antigen was found for all PLGpS microparticle formulations (>70%). This result is consistent with our findings that the initial concentration of protein solution can dramatically effect the encapsulation efficiencies.

The mass of microparticles administered was adjusted such that the required dose of 10.0 μg of Flu (trivalent - 2.35 μg total HA) was delivered. No attempt to optimize the dose of adjuvant coadministered was made, although it is expected that this would be a function of formulation conditions.

The mice showed no gross pathologies or behavioral changes after immunization with PLGpS microparticles that contained encapsulated Flu (trivalent) vaccine or Flu (trivalent) vaccine and adjuvants cocktails. were obtained on days 21, 42 and 57 and were evaluated for total IgG, IgG1, IgG2a and for the presence of functional antibody via the hemagglutination inhibition antibody assay (HAI). Each strain (A/Texas, A/Johannesburg and B/Harbin) was examined to evaluate the effects of encapsulation and release immunogenicity and functional antibody in a multicomponent system. All samples were analyzed duplicate. The antibody ELISA's were performed as described in Example 13.

Figures 14a to c, show the specific IgG antibody responses elicited by PLGpS/Flu (trivalent) microparticle formulations, to each strain contained within the Flu (trivalent) vaccine.

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By day 57, the highest total IgG titres were found PLGpS/Flu (trivalent)/DC-Chol microparticle This result was true for each strain formulations. (A/Texas -32x response οf soluble control, A/Johannesburg - 64x response of soluble Flu control, B/Harbin - 64x response of soluble Flu control).

By day 57, slightly higher IgG antibody responses, as indicated by titre, to A/Texas (2x response of soluble Flu control) and A/Johannesburg (4x response of soluble Flu control) were found for PLGpS/Flu(trivalent)/PCPP microparticle formulations.

By day 57, essentially identical total specifical graph of the planting of strain, as indicated by titre, were elicited for the planting (trivalent), planting (trivalent), planting (trivalent), planting of the soluble flu (trivalent) vaccine formulations and the soluble flu (trivalent) vaccine control group. This is consistent with the results obtained in Example 13 with monovalent vaccine where similar results were observed for these three systems.

To determine the influenza hemagglutination inhibition antibody assay (HAI) for each HA strain, serum samples were heated at 56°C for 30 inactivate complement and pre-treated with trypsin (0.1 mL of 8 mg/mL)/periodate (12.5 μ L - 0.001M) to destroy endogenous inhibitors of hemagglutination. diluted antisera were tested in duplicate for their ability to inhibit the agglutination of 1% chick red blood cells by 4 HAU of A/Texas, A/Johannesburg or B/Harbin virus respectively in a standard HAI assay (ref. 33).

Figures 15a to c, show the specific HAI titres elicited to each HA strain contained within the trivalent vaccine. By day 57, the highest sustainable

HAI titres were found for PLGpS microparticles formulated with Flu (trivalent) vaccine in the presence of DC-Chol (HAI 240 to HAspecific A/Texas (8X soluble (trivalent) control), 240 to HA specific A/Johannesburg (16X soluble Flu (trivalent) control) and 60 to HA specific B/Harbin (8X soluble Flu (trivalent) control)). higher titres were also found for PLGpS micropartilcles formulated with Flu (trivalent) in the presence of BAY (HAI titre; 60 to HA specific A/Texas (2X soluble Flu (trivalent) control), 60 to HA specific A/Johannesburg (4X soluble Flu (trivalent) control) and 15 to HA specific B/Harbin (equal to soluble Flu (trivalent) control)). The HAI titres found for BAY formulations with Flu (trivalent) vaccine, in this Example, are comparable to those obtained for the low dose groups in Example 13, wherein 1 μg Flu (monovalent) vaccine - 0.325 μ g A/Texas specific HA was administered. (trivalent)/PCPP microparticle formulations failed to elicit any detectable functional antibody response.

An experiment was conducted examining a higher dose of PLGpS/Flu (triv)/DC-Chol formulated microparticles. This group of mice was immunized on day 1 with approximately twice the dose of Flu (triv) vaccine administered to the other groups (Dose - Flu(triv) = 21.2 μ g, total HA = 5.00 The results obtained for this high dose are included in Figures 15a to c. The HAI titres elicited for a control group immunized with soluble Flu (triv) vaccine at this dose were marginal and essentially equivalent to the low dose group examined (results not shown). By day 57, formulation elicited significantly higher specific HAI titres than the low dose group (HAI titre; 480 to specific A/Texas (5X soluble Flu (trivalent)

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control), 480 to HA specific A/Johannesburg (5X soluble Flu (trivalent) control) and 120 to HA specific B/Harbin (3X soluble Flu (trivalent) control). Additionally, it was found that the HAI titres elicited were sustainable, being maintained at these levels from day 21 through 57.

Additives, such as BAY or DC-Chol, were introduced into the organic phase, containing polymer and organic solvent wherein they can function at the interface to protect antigen during formulation procedures (see Example 13, Table 8). This serves to increase the recovery of antigenic material.

In addition, in vitro release studies have shown that a sizable amount of the encapsulated components are released within the first three days. It has been experimentally determined that about 5 to 15% of antigen encapsulated is detectable at this stage of release (antigen which is surface localized on the microparticle). The priming immunization was significantly enhanced by the co-release of adjuvants. This is likely the result of being localized in close proximity to antigen during the initial release phase.

Ιt is noteworthy that PLGpS microparticle · formulations coencapsulating antigens with adjuvants, such as PCPP, did not result in formulations with improved vaccine efficacy. This material was added to the aqueous phase of the primary emulsion and does not possess the lipophilic properties of DC-Chol or BAY. It has been demonstrated that PCPP has strong adjuvant properties in other systems, yet in this Example, a marginal improvement in encapsulation efficiency or adjuvancy of the microparticle formulation is observed.

The studies presented in this Example demonstrate that viral antigens from influenza virus in a multi-

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component system can elicit high levels of total IgG antibody and functional antibodies (HAI) when the Flu antigens are entrapped in microparticles in the presence of an additional co-encapsulated adjuvant, such as BAY or DC-Chol.

Example 16:

This Example evaluates the infection rate and protection after single dose subcutaneous immunization with PLGpS microparticle formulations in the mouse model.

A/Taiwan/1/86 (H1N1) as live influenza viruses in egg-derived allantoic fluid, mouse-adapted A/Taiwan/1/86 and commercial A/Taiwan/1/86 monovalent subunit vaccine (Fluzone®) were obtained from Pasteur Mérieux, Connaught, USA (Swiftwater, PA).

Groups of 8 female DBA-2 mice aged 6 to 8 weeks (described in Example 14) were immunized subcutaneously day 1 with Flu (trivalent) vaccine containing A/Texas, A/Johannesburg and B/Harbin strains (2.35 μg total HA in 0.25 mL PBS). Control mice received either PBS alone or 400 hemagglutination units (HAU) of live A/Texas virus as allantoic fluid. Fourteen days later, mice were challenged intranasally while under anesthesia with 50 μL of live mouse-adapted A/Taiwan/1/86 (5 LD50) in allantoic fluid. Protection assessed by monitoring mortality daily morbidity (weight change) every 2 days up to 14 days post-challenge.

Figure 16 shows the results of the challenge. As expected, all mice immunized with homologous live (A/Texas) virus suffered minimal loss in weight and fully recovered within 2 weeks of challenge as measured

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by the weight percent change from baseline. Also as expected, mice immunized with PBS suffered a precipitous drop in weight which by day 10 fell below 30% of baseline. No mice in this control group survived after 10 days.

Mice immunized with PLGpS/Flu (trivalent)/DC-Chol microparticle formulations were successfully infected, as indicated by the 8% average drop in weight within 4 days post challenge. Of the PLGpS formulated groups examined, these mice exhibited the swiftest recovery, reaching 100% of baseline after 2 weeks. The entire group recovered indicating a protective titre was raised in each mouse.

Mice immunized with PLGpS/Flu (trivalent)/BAY microparticle formulations were also successfully infected, as indicated by the 10% average drop in weight by day 10. These mice recovered more slowly, reaching 95% of baseline after 2 weeks. The entire group recovered indicating that a protective titre was raised in all of these mice, although the rate of recovery indicates the level of protection was less than that seen for the DC-Chol group at this dose. This is in accordance with the HAI values determined in Example 15 for these two groups.

The soluble Flu (trivalent) vaccine control group and the PLGpS/Flu (trivalent) or PLGpS/Flu (trivalent)/PCPP microparticle formulations were less successful. These groups experienced a 23 to 29% average drop in weight by days 8-10 post challenge. The rate of recovery was slowest for these mice reaching 86 to 92% of baseline after 2 weeks. Additionally not all mice in these groups survived. Six of eight mice survived from the PLGpS/Flu (trivalent) microparticle formulated group. Seven of eight survived from the

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PLGpS/Flu (trivalent)/PCPP formulated group and six of eight survived from the soluble Flu (trivalent) control group. In each of these cases no or low protection is observed.

5 In Example 14, we evaluated the effects microencapsulation formulation conditions on the Flu (trivalent) vaccine. Strain-specific (A/Texas, A/Johannesburg B/Harbin) and analysis of antigen SRID suggested that minimal recovery by loss 10 antigenic activity for all three strains in this multicomponent system could be expected when employing DC-Chol or BAY in the organic phase with PLGpS polymer and EtOAc as solvent.

In Example 15, the higher functional antibody (a correlate for protection) elicited by single subcutaneous administration of PLGpS/Flu (trivalent) vaccine formulated in the presence of DC-Chol or BAY demonstrated the utility of these formulations in the The results of the challenge/protection mouse model. study, described in Example 16, are in agreement with ranking for protection as suggested by the functional antibody studies.

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The delivery of antigen and adjuvant within a biodegradable microparticle can result in more efficient presentation to the immune system. The adjuvancy found for soluble mixtures of DC-Chol or BAY with antigen(s) may be significantly increased by employing this formulation strategy. The possibility for fewer and lower (antigen/adjuvant) dose regimens is indicated by these results. Specifically, this Example strongly indicates the potential of these microparticle based formulations for development as a single efficacious dosage form.

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Example 17:

This Example illustrates the immunogenicity of transferrin binding protein (Tbp-2) derived from Moraxella catarrhalis (as described in WO 97/13785, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference) encapsulated in PLGpS microparticles, Tbp-2 physically mixed with PLGpS microparticles or Tbp-2 formulated with Alum in mice immunized subcutaneously.

Groups of five, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized subcutaneously (S.C.) with the following amounts of antigen in 250 μL of PBS (pH 7.4) on days 1, 28 and 43: PLGpS microparticles prepared as described in Example 5 containing 0.3 μg of Tbp-2; PLGpS microparticles prepared as described in Example 5 physically mixed with 0.3 μg of Tbp-2; and Alum (1.5 mg/dose) formulated with 0.3 μg of Tbp-2 (Table 5).

The mice showed no gross pathologies or behavioral changes after receiving microparticles that contained encapsulated Tbp-2, microparticles physically mixed with Tbp-2 or Alum physically mixed with Tbp-2. Sera were collected on days +14, +27, +42 and +55 and were evaluated for the presence of anti-Tbp-2 IgG antibodies by antigen specific ELISA as described in Example 8. All samples were analysed in duplicate.

The core loading of PLGpS microparticles containing tbp-2 was determined via amino acid analysis (4.0 μ g/mg (32.8%)) as described in Example 6.

The results of immunizations (Figure 17a) indicate that antigen presented to the immune system entrapped in PLGpS microparticles elicited a substantially higher titre than that obtained for soluble antigen or for

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antigen physically mixed with PLGpS microparticles alone. In addition, this study indicates that microencapsulated formulations were as immunogenic as the traditional Alum adjuvanted systems. The kinetics of the immune response were found to be similar for both formulations.

The IgG subtype profile (Figure 17b) for the bleed obtained on day 55 revealed differences in the immune responses elicited for Tbp-2 encapsulated in PLGpS microparticles and Tbp-2 physically mixed with PLGpS microparticles or formulated with Alum. IgG1 is the dominant subtype detected (with some IqG2b) antigen was administered in soluble form or as physical mixture with PLGpS microparticles formulated with Alum.

In this Example, the IgG subtypes induced by Tbp-2 encapsulated within PLGpS microparticles elicit comparatively more IgG2a. This trend is a similar trend to that observed for Hin-47 in Example 8.

These results suggest that the mechanisms of immune response induced by immunizing with encapsulated antigens are different from that generated with Alum. A more balanced Th_2/Th_1 profile (as indicated by the IgG2a:IgG1 ratio) is exhibited when antigen is administered encapsulated within microparticles.

As may be seen from the results herein, quality of the response mediated immune by PLGpS microparticles encapsulating antigen is substantially different from that obtained by physically mixing with PLGpS microparticles, formulating with Alum or by administering soluble antigen alone. Additionally, the magnitude of the immune response induced by Alum in antigen/Alum formulations is comparable to that

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provided by microparticles containing encapsulated antigen.

Example 18:

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This Example illustrates the immunogenicity of Tbp-2 encapsulated in PLGpS microparticles and Tbp-2 physically mixed with PLGpS microparticles in mice immunized intranasally.

Groups of five, 6 to 8 week old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were immunized intranasally (I.N.) with the following amounts of antigen in either 10 µL or 50 µL of PBS (pH 7.4) on days 1, 28 and 43: PLGpS microparticles prepared as described in Example 5 containing 6.0 µg of Tbp-2; and PLGpS microparticles prepared as described in Example 5 physically mixed with 6.0 µg Tbp-2 (Table 5).

The mice showed no gross pathologies or behavioral changes after immunization with PLGpS microparticles that contained encapsulated Tbp-2 or PLGpS microparticles that were physically mixed with Tbp-2. Sera were obtained on days 14, 27, 42 and 55 and were evaluated for the presence of anti-Tbp-2 IgG antibodies by antigen specific ELISA as described in Example 8. All samples were analyzed in duplicate.

The core loading of PLGpS microparticles containing Tbp-2 was determined via amino acid analysis (4.0 μ g/mg (32.8%)) as described in Example 6.

The serum IgG Tbp-2-specific antibody titres following I.N. immunization is shown in Figure 18. When the volume of the dose administered was low (10 μ L) these results indicate that an antigen (Tbp-2) incorporated into or physically mixed with PLGpS

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microparticles is less immunogenic than soluble antigen of similar dose (6.0 μg).

When the volume of the dose was increased (50 μL), the overall titres are significantly higher for all groups. These results indicate that an antigen (Tbp-2) physically mixed with PLGpS microparticles substantially more immunogenic than microparticle encapsulated antigen or for soluble antigen of similar dose (6.0 μg) in accordance with earlier observations made for intranasal immunization of Hin-47 (Example 10).

In our initial study with intranasal immunizations, Example 10, it was found that a strong humoral response and robust secretory response were obtained by administering Hin-47 physically mixed with microparticles. This study with Tbp-2 has confirmed the earlier observations. Additionally, we established that the volume of the dose administered plays a significant role in the type and strength of the immune response invoked.

Example 19:

This Example illusrates the release of antigen from the microparticles.

Having established that antigen encapsulated within PLGpS microparticles is as immunogenic as Alum formulations, we next sought to examine whether the prime and delayed release character of antigens encapsulated within these polymeric matrices could be exploited so that fewer immunizations would be needed.

In this Example, we examined the immunogenicity of Tbp-2 encapsulated in PLGpS microparticles, physically mixed with PLGpS microparticles or formulated with Alum or CFA/IFA in guinea pigs immunized subcutaneously.

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Groups of two, 6 to 8 week old female guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) were immunized subcutaneously (S.C.) with the following amounts of antigen: PLGpS microparticles encapsulating 5.0 μg of tbp-2 suspended in 500 μL of PBS (pH 7.4) on days 1 and 28, prepared as described in Example 5; Complete Freunds Adjuvant (CFA) formulated in PBS (pH 7.4)with μg of Tbp-2 5.0 on day 1 intramuscularly (I.M.) by Incomplete Freunds Adjuvant (IFA) formulated in PBS (pH 7.4) with 5.0 μg of Tbp-2 on days 14 and 28; or Alum formulated in PBS (pH 7.4) with 5.0 μ g of Tbp-2 on days 1, 14 and 28 (Table 5).

The guinea pigs showed no gross pathologies or behavioral changes after immunization with **PLGpS** microparticles that contained encapsulated Tbp-2, PLGpS microparticles physically mixed with Tbp-2, CFA/IFA formulated Tbp-2 or Alum formulated Tbp-2. Sera were obtained on days 40 and 56 and were evaluated for the presence of anti-Tbp-2 IgG antibodies by antigen specific ELISA. All samples were analyzed duplicate. The antibody ELISA's are described in Example 8.

The core loading of PLGpS microparticles containing Tbp-2 was determined via amino acid analysis (4.0 μ g/mg (32.8%)) as described in Example 6.

The results shown in Figure 19 indicate that two doses of PLGpS microencapsulated Tbp-2 elicited similar anti-Tbp-2 antibody responses as 3 doses of Tbp-2 formulated either in CFA/IFA or with Alum.

The present results strongly suggest that microencapsulated Tbp-2 may be exploited as a candidate vaccine for two dose schedules.

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Example 20:

This Example illustrates the immunogenicity of rUrease or rUrease/adjuvant cocktails encapsulated within PLGpS microparticles in mice immunized subcutaneously or intragastrically.

Groups of eight, 6 to 8 week old outbred Swiss female mice (Janvier, France) were immunized subcutaneously (S.C.) with the following amounts of antigen in 300 μL of PBS (pH 7.4) on days 1 and 28 and PLGpS microparticles prepared as described Example 5 containing 10.0 μg of rUrease; and PLGpS microparticles prepared as described Example in coencapsulating 10.0 µg of rUrease and DC-Chol, PCPP or CT-X; 10.0 μg of rUrease plus soluble DC-Chol (65 μ q/dose) or PCPP (100 µg/dose) as controls intragastrically (I.G.) with the following amounts of antigen in 300 μL of 0.15 M NaHCO3 (pH 9.0) on days 1, 28 and 56: PLGpS microparticles prepared as described in Example 5 containing 40.0 µg of rUrease; and PLGpS microparticles prepared as described in Example coencapsulating 40.0 µg of rUrease and DC-Chol, PCPP or CT-X (Table 5).

The core loading οf PLGpS microparticles containing rUrease was determined via amino analysis or polyclonal rUrease specific ELISA (Table 7) as described in Example 6. The polyclonal ELISA assay performed on PLGpS microparticle extracts typically provides a measure of total protein (recovered epitope) encapsulated. Control experiments have allowed us to quantify the extent to which the solvent/base (SDS) extraction procedure effects the integrity of the antigen. Under the conditions employed we have estimated that about 65 to 75% of the extracted protein

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remains fully detectable by this assay. Thus this determination will be lower than the measure of total protein recovered via amino acid analysis (AAA). Suitable controls were included to ensure that the presence of the adjuvants (DC-Chol, CT-X or PCPP) did not interfere with the results obtained. The presence of DC-Chol did not seem to influence the however, PCPP provided anomalous values which were uncharacteristically higher when compared to the total protein by AAA in some analyses. In the case of CT-X, a separate polyclonal ELISA assay was developed to quantify the amount of CT-X coencapsulated.

The mass of microparticles administered was adjusted such that the required dose of 10.0 μg of rUrease (subcutaneously) or 40.0 μg of rUrease (orally) was delivered.

The mice showed no gross pathologies or behavioral changes after immunization with PLGpS microparticles that contained either encapsulated rUrease or coencapsulated mixtures of rUrease and adjuvant. Sera were obtained on day 85 and were evaluated for the presence of anti-rUrease IgG1 and IgG2a antibodies by antigen specific ELISA. All samples were analyzed in duplicate.

25 were performed according to ELISA's standard protocols (biotinylated conjugates, streptavidine peroxidase complex Amersham were from phenylenediamine dihydrochloride (OPD) substrate from Sigma). Plates were coated overnight at 4° C with H. 30 pylori extracts (5 μg/mL) in 0.05Mcarbonatebicarbonate buffer (pH 9.6). After saturation with BSA (Sigma), plates were incubated with sera (1.5 hrs), biotinylated conjugate (1.5)hrs), strepavidin peroxidase complex (1h) and substrate (OPD - 10 min).

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A polyclonal mouse serum directed against H. pylori extract served as a control in each experiment. The titres were expressed as the inverse of the dilution giving 50% of the maximal absorbance value at 492 nm. Pre-immune sera is used as negative control.

Mice were immunized by the subcutaneous or intragastric route in the presence of adjuvants chosen based on Th_2/Th_1 profile (DC-Chol - more balanced Th_2/Th_1 , PCPP - primarily Th_2) or for known mucosal adjuvancy (Cholera Toxin (CT-X) or heat labile enterotoxin from $E.\ coli\ (LT)$). PLGpS microparticles have been shown to induce a more balanced Th_2/Th_1 profile relevant to other typical adjuvants, such as Alum.

15 The IgG subtype profile for pooled bleeds obtained on day 85 after subcutaneous immunizations is shown in Figure 20. In all cases, the IgG1 response was higher than the IgG2a response (IgG2a: IgG1 ratio ranges from to 0.33) when PLGpS/rUrease microparticle formulations were administered subcutaneously. 20 total IgG1 response for the soluble control groups (rUrease + DC-Chol or PCPP) was in the same absolute There are differences noted for the IgG2a range. responses between adjuvant systems that were either 25 coencapsulated with antigen or administered а physical mixture with antigen.

For PLGpS/rUrease microparticles (IgG2a:IgG1 = 0.33), PLGpS/rUrease/CT-X (IgG2a:IgG1 = 0.30) and PLGpS/rUrease/DC-Chol (IgG2a:IgG1 = 0.20), a more balanced IgG2a:IgG1 antibody response (indicative of Th_2/Th_1 ratio) was obtained. For comparison, the rUrease + DC-Chol control group IgG2a:IgG1 = 0.03.

For PLGpS/rUrease/PCPP microparticle formulations, primarily a Th_2 response is noted IgG2a:IgG1 ratio =

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0.08. The analogous soluble mixture of rUrease + PCPP is strongly Th_2 biased with a very low IgG2a:IgG1ratio = 0.003.

In general, coencapsulation of antigen in the presence of these adjuvants tends to shift the typical immunological profile towards a more balanced Th_2/Th_1 response.

Immunization via the intragastric route in most cases did not elicit any detectable systemic response. Notable exceptions to this are the positive control 10 with LT having a modest systemic response (IgG2a:IgG1 ratio = 0.1and for rUrease/PLGpS microparticles coencapsulating CT-X(IgG2a:IgG1 ratio Interestingly, for oral immunizations, encapsulated antigen plus mucosal adjuvant induces a strikingly 15 different systemic antibody response. The ratio of IgG2a:IgG1 is now strongly in favor of indicative of the Th₁ path. Examination of the that CT-X co-administered literature reveals antigen orally typically induces an immune response similar to that elicited by LT. Primarily an IgG1 or TH₂ type of response is reported (ref. 34). This study illustrates that the quality of the immune response may be changed as a consequence of coencapsulation in PLGpS microparticles.

Example 21:

This Example evaluates the infection rate protection after subcutaneous or oral immunizations with PLGpS microparticle/rUrease formulations in the mouse model.

Mice were challenged 6 weeks after the 2'nd boost (Day 85) by gastric gavage with 300 μL of a suspension of H. pylori bacteria (3x106 cfu). Based on in vitro

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release studies (Example 7), antigen release from microparticles requires approximately 4 weeks, thus the challenge was scheduled for 2 weeks after this time point.

Four weeks after the challenge, mice were killed and stomachs were sampled to evaluate urease activity (Jatrox test, Procter and Gamble) in a sterile flow hood. Urease activity was assessed 24 hours postmortem by measuring the absorbance at 550 nm. The principle of the test is that the urea present in the test medium is split by Helicobacter pylori urease. The rise in pH causes a color change in the indicator which is likewise present in the test medium (phenol red), from yellow to pink red.

15 Mice were infected with a streptomycin-resistant mouse-adapted strain (X43-2AN) of H. pylori. The infection rate was reproducible; 100% of the mice were infected in all experiments, as judged by urease activity measured in the stomach (Jatrox test). to be noted that the streptomycin resistance of the 20 challenge strain allows it to grow on selective medium, making this test very sensitive (no contaminants coming from the normal flora).

This strain was stored at -70°C in Brucella Broth (Biomérieux) supplemented with 20% v/v glycerol and 10% v/v foetal bovine serum (FBS) (Hyclone). challenge suspension was prepared as follows: for preculture, H. pylori was grown on Mueller-Hinton Agar (MHA; Difco) containing 5% v/v sheep blood (Biomerieux) and antibiotics: Thrimethoprim (5 $\mu g/mL$), Vancomycin (10 μq/mL), Polymixin В sulphate (5 $\mu g/mL)$, Amphotericin (5 μg/mL) and Streptomycin (50 μg/mL) selective marker of H. pylori strain X43-2AN (TVPAS). All antibiotics were purchased from Sigma. MHA-TVPAS

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plates were incubated for 3 days at 37°C under microaerobic conditions. The pre-culture was used innoculate a 75 cm² vented flask (Costar) containing 50 supplemented with 5% v/v FBS and all antibiotics (TVPAS). The flask was kept under microaerobic conditions with gentle shaking for 24 hrs. suspension was characterized by Gram's staining, urease activity (Urea indole medium, Diagnostic Pasteur), 3% catalase $(H_2O_2,$ v/v) and oxidase activity (Biomerieux discs). Viability and motility were checked by phase contrast microscopy. The suspension was diluted in BB to O.D. 550 nm = 0.1 (which was equivalent to 1 \times 10⁷ CFU/mL).

In this study, mice for which an OD. < 0.1 was obtained were considered protected or at least have less than 10^3 to 10^4 bacteria/stomach (compared to 10^5 to 10^6 in controls). Groups of mice representing unimmunized/infected and unimmunized/uninfected controls were also included in the study (results not shown).

Figure 21a, shows that protection (via the subcutaneous route), as judged by the assay described above, follows the ranking PLGpS/rUrease/DC-Chol (geometric mean = 0.092) > PLGpS/rUrease/CT-X (geometric mean = 0.105), PLGpS/rUrease (geometric mean 0.163)and PLGpS/rUrease/PCPP (geometric mean = 0.269). The LT + rUrease (oral) positive control group (geometric mean = 0.136) is the standard for protection in this study. Interestingly, typical results with rUrease plus soluble DC-Chol (geometric mean = 0.600) or rUrease plus PCPP (geometric mean = 1.07) control groups clearly show the advantages of coencapsulating antigen and adjuvant PLGpS as a microparticle formulation.

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Mann-Whitney statistical analysis of the presents the following conclusions. The PLGpS/rUrease/DC-Chol and PLGpS/rUrease/CT-X formulations were not significantly different from the rUrease positive control group. The PLGpS/rUrease/PCPP, rUrease + DC-Chol and rUrease + PCPP groups were significantly different from these (p < 0.01) and the PLGpS/rUrease group was significantly different from the PLGpS/rUrease/PCPP, rUrease + DC-Chol and the rUrease + PCPP (p < 0.01) groups.

From this analysis, the PLGpS/rUrease/DC-Chol (7/8 mice have OD. < 0.1) and PLGpS/Urease/ CT-X (5/8 mice have OD. < 0.1) microparticle groups exhibited solid protection whereas the PLGpS/rUrease (2/8 mice have OD. < 0.1) microparticle group and the rUrease + DC-Chol group (2/10 OD. < 0.1) exhibited moderate protection and the PLGpS/rUrease/PCPP (0/8 mice have OD. < 0.1) microparticle group and the rUrease + PCPP groups (0/10 OD. < 0.1) exhibited low or no protection respectively. This ranking tends to follow the more balanced Th_2/Th_1 ratios as determined from immunogenicity studies (Example 20).

Figure 21b shows that no full protection via the oral route was observed for all groups of mice immunized by PLGpS microparticles containing additional adjuvants.

Statistical ranking follows the order PLGpS/rUrease/CT-X (geometric mean = 30 PLGpS/rUrease/DC-Chol (geometric mean PLGpS/rUrease (geometric mean 0.475)PLGpS/rUrease/PCPP (geometric mean = 0.493). The LT + rUrease (oral) group (geometirc mean = 0.136) is the positive control for this study.

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Mann-Whitney statistical analysis of the data presents the following conclusions. The LT + rUrease positive control group was significantly different than the PLGpS/rUrease/DC-Chol and PLGpS/rUrease/CT-X formulations (p < 0.01). The PLGpS/rUrease/DC-Chol and PLGpS/rUrease/CT-X formulations were not significantly different from each other. The PLGpS/rUrease and the PLGpS/rUrease/PCPP formulations were not significantly different form each other.

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In this study, the PLGpS/rUrease/CT-X (2/8 mice have OD. < 0.1) and the PLGpS/rUrease/DC-Chol (1/8 mice have OD. < 0.1) microparticle formulations exhibited partial protection. Comparatively, the positive control group of rUrease plus LT exhibited solid protection (7/8 mice have OD. < 0.1).

It was suggested, in Examples 14 to 16, that the presentation of adjuvant to the immune system by association with delivery vehicles, such as PLGpS, microparticles increases the adjuvants effectiveness resulting in a more efficacious vaccine.

Notably, typical control studies in this Example have examined immunogenicity and protection after immunizations with soluble mixtures of adjuvants, such as DC-Chol or PCPP, and rUrease by the subcutaneous route, and have found that protection was These results provide additional evidence moderate. that the presentation of antigen and adjuvant in the of particulated а matrix can result substantially more efficacious vaccines.

Additional benefits, such as reduced adjuvant toxicity and the possibility of modulating the quality of the immune response which is characteristic for the adjuvant employed, have been demonstrated in this Example. It was experimentally determined that the IgG

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subtype antibody responses to rUrease obtained after oral immunization with PLGpS/rUrease/CT-X formulated microparticles was uncharacteristically in favor of IgG2a (Th_1 path).

In the case of coencapsulated antigen and CT-X (a known mucosal adjuvant) administered subcutaneously or orally, it is also likely that this material stabilizes the antigen within the polymeric matrix during formulation, storage and release by a mechanism similar to that observed for microparticles prepared in the presence of HSA or BSA (ref. 35).

In conclusion, this study demonstrates the feasibility and efficacy of PLGpS microparticle based systemic immunization to induce significant protection against *H. pylori* infection in the mouse model. This study also demonstrates the feasibility for PLGpS microparticle based oral immunization to induce partial protection against *H. pylori* infection in the mouse model.

Additionally, co-encapsulation of rUrease in the presence of additional adjuvants (specifically DC-Chol or CT-X), can result in notable improvement in vaccine efficacy.

25 SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides a particulate carrier for an agent, particularly one having biological activity, comprising a matrix of polymer and biologically active material.

The particulate carriers in the form of microparticles are able to efficiently deliver agents to the cells of the immune system of a subject following mucosal or parenteral administration to produce an immune

response. Modifications are possible within the scope of this invention.

TABLE 1

Antigen(s) entrapped in PLG, PLGpZS or PLGpS microparticles	Antigen Preparation
Hin-47 (1.55 mg/mL) + BAY (5.0 mg/mL)	800 µL of Hin-47 in aqueous internal phase plus 40.0 mg of BAY in the organic phase
rD-15 (2.05 mg/mL)	800 µL of rD-15.
Hin-47 (1.95 mg/mL) + rD-15 (1.95 mg/mL)	400 μL of Hin-47 plus 400 μL of rD-15.
Flu X-31 (2.0 mg/mL) Flu A-Texas (1.48 mg/mL)	800 μL of Flu X-31 or 800 μL of Flu A-Texas
Flu (2.0 mg/mL) + BAY (5.0 mg/mL) Flu A-Texas (1.48 mg/mL) + BAY (5.0 mg/mL)	800 µL of Flu X-31 or 800 µL of Flu A-Texas in aqueous internal phase plus 40.0 mg of BAY in the organic phase

TABLE 2

Summary of Microparticle Core Loading and Encapsulation Efficiencies (Hin-47, Hin-47 + Bay R1-005)

Protein	Polymer	Epitope Equiv.	Total Protein	Epitope	Total Adjuvant
(Conc.)		HIN-47	via AAA²	vs Total	via AAA²
(Adjuvants)		via ELISA¹-	(Encaps. Eff.) ³	Protein	(Encaps. Eff.)
		(Encaps. Eff.) ³		8	
	PLG	1.4 ug/mg	2.8 ug/mg	50%	
		(10.2%)	(20.4%)		
Hin-47	PLGpZS	5.3 ug/mg	7.5 ug/mg	71%	
(1.55 mg/mL)		30.8%	(43.5%)		
				•	
	PLGpS	1.6 ug/mg	3.3 ug/mg	48%	
		(11.6%)	(23.9%)		, .
	PLG	2.5 ug/mg	3.8 ug/mg	66%	13.5 (3.4%)
		(20.1%)	(30.6%)		
Hin-47					;
(1.55 mg/mL)	PLGpZS	4.1 ug/mg	5.5 ug/mg	75%	15.6 (3.9%)
Bay-R1005		(19.8%)	(26.6%)		
(5.0 mg/mL)					
	PLGpS	6.1 ug/mg	9.2 ug/mg	66%	23.2 (4.6%)
		(39.4%)	(59.4%)		

- ELISA values are averages of 2 independent measurements of protein obtained after complete hydrolysis of microparticles.
- 2) AAA values are averages of 2 independent measurements on protein (or adjuvant) encapsulated within microparticles and 2 independent measurements on protein (or adjuvant) obtained after complete hydrolysis of microparticles.
- 3) Encapsulation efficiency (Encaps. Eff.) calculated as follows:

 total mass of protein (or adjuvant) recovered X 100% total amount of protein (or adjuvant) used

TABLE 3

Summary of Microparticle Core Loading and Encapsulation Efficiencies (rD-15, rD-15 + Hin-47)

Protein	Polymer	Pritone Provinciant	
11000111	Lordwer	Epitope Equivalent	Total Protein
(Conc.)		Hin-47 via ELISA¹	via AAA²
		(Encaps. Eff.) ³	(Encaps. Eff.) ³
	PLG		7.8 ug/mg (43.7%)
rD-15			
		•	
(1.95 mg/mL)	PLGpZS		7.1 ug/mg (39.2%)
	PLGpS		7.3 ug/mg (43.7%)
		·	
rD-15	PLG	1.6 ug/mg (8.0%)	7.0 ug/mg (35.1%)
(1.95 mg/mL)			
+ Hin-47	PLGpZS	5.5 ug/mg (26.1%)	13.1 ug/mg (62.1%)
	•		ag,g (02.10)
(1.95 mg/mL)			
	PLGpS	2.4 ug/mg (10.8%)	(11.6 ug/mg (52.1%)
	<u>F</u>		(ug/mg (()

- 1) ELISA values are averages of 2 independent measurements of protein obtained after complete hydrolysis of microparticles.
- 2) AAA values are averages of 2 independent measurements on protein encapsulated within microparticles and 2 independent measurements on protein obtained after complete hydrolysis of microparticles.
- 3) Encapsulation efficiency (Encaps. Eff.) calculated as follows:

 total mass of protein recovered X 100%
 total amount of protein used

TABLE 4

Summary of Microparticle Core Loadings and Encapsulation Efficiencies for Flu X-31 + Bay R1-005 and Flu A-Texas, Flu A-Texas + Bay R1-005

Protein	Polymer	Total Protein	Total Adjuvant
(Conc.)		via AAA¹	via AAA¹
(Adjuvants)		(Encaps. Eff.) ²	(Encaps. Eff.) ²
	PLG	4.7 ug/mg (26.1%)	
Flu X-31 (2.0 mg/mL)	PLGpZS	6.1 ug/mg (34.1%)	
	PLGpS	7.1 ug/mg (39.7%)	
	PLG	5.6 ug/mg (35.1%)	22.9 ug/mg (5.2%)
Flu X-31 (2.0 mg/mL Bay R1005 (5.0 mg/mL)	PLGpZS PLGpS	8.4 ug/mg (52.5%) 5.0 ug/mg (31.0%)	28.1 ug/mg (10.0%) 27.4 ug/mg (8.4%)
Flu-A-Texas (1.48 mg/mL)	PLGpS	2.7 ug/mg (22.8%)	
Flu A-Texas (1.48 mg/mL) Bay-R1005 (5.0 mg/mL)	PLGpS	3.4 ug/mg (31.6%)	11.5 ug/mg (3.5%)

¹⁾ AAA values are averages of protein (or adjuvant) isolated after complete hydrolysis of microparticles and protein (or adjuvant) encapsulated within microparticles.

2) Encapsulation efficiency (Encaps. Eff.) calculated as follows:

total mass of protein (or adjuvant) recovered X 100% total amount of protein (or adjuvant) used

Summary of Microparticle Formulation Procedures

TABLE 5

Antigen(s)/Adjuvants entrapped in PLGpS microparticles	Antigen Preparation
tbp-2 (2.88 mg/ml)	800 µl tbp-2 (diluted to 1.44 mg/ml) in aqueous phase with PBS
Flu (Trivalent) (0.533 mg/ml)	800 µL Flu (trivalent) in aqueous internal
Flu (Trivalent) (0.533 mg/ml) + BAY (3.3 mg/ml)	800 μL Flu (trivalent) in aqueous internal phase 40.0 mg of BAY in the organic phase
Flu (Trivalent) (0.533 mg/ml) + DC-Chol (3.3 mg/ml)	800 µL Flu (trivalent) in aqueous internal phase 40.0 mg of DC-Chol in the organic phase
Flu (Trivalent) (0.533 mg /ml) + PCPP (1.25 mg/ml)	800 µL of combined solution in aqueous internal phase
rUrease (1.00 mg /ml) rUrease (1.00 mg /ml) + CT-X (0.5 mg/ml) rUrease (1.00 mg /ml) + DC-Chol (3.3 mg/ml) rUrease (1.00 mg /ml) +	800 μL rUrease in aqueous internal phase 800 μL of combined solution in aqueous internal phase 800 μL rUrease in aqueous internal phase 40.0 mg of DC-Chol in the organic phase
PCPP (1.25 mg/ml)	800 µL of combined solution in aqueous internal phase

Table 6

Summary of Microparticle Core Loadings and Encapsulation Efficiencies for Flu (trivalent) and Flu (trivalent) + Adjuvants

Protein (Conc.) (Adjuvants)	Polymer	Total Protein via AAA¹ (Encapsulation Efficiency)²
Flu (trivalent) (0.533 mg/mL)	PLGpS	5.0 ug/mg (93.3%)
Flu (trivalent) (0.533 mg/mL) Bay-R1005 (3.3 mg/mL)	PLGpS	5.8 ug/mg (104.6%)
Flu (trivalent) (0.533 mg/mL) DC-Chol (3.3 mg/mL)	PLGpS	3.9 ug/mg (72.7%)
Flu (trivalent) (0.533 mg/mL) PCPP (1.25 mg/mL)	PLGpS	4.6 ug/mg (86.6%)

- 1) AAA values are averages of protein isolated after complete hydrolysis of microparticles and protein encapsulated within microparticles.
- 2) Encapsulation efficiency calculated as follows: total mass of protein recovered X 100% total amount of protein used

Table 7

Summary of Microparticle Core Loadings and Encapsulation Efficiencies for rUrease and rUrease + Adjuvants

Protein (Conc.) (Adjuvants)	Polymer	Total Protein via AAA¹ (Encaps. Eff.)³	Total Protein via ELISA ² (Encaps. Eff.) ³
rUrease (1.0 mg/mL)	PLGpS	3.44 ug/mg (45.8 %)	2.5 ug/mg (33.2 %)
rUrease (1.0 mg/mL) DC-Chol (3.3 mg/mL)	PLGpS	3.15 ug/mg (44.0 %)	2.2 ug/mg (30.3 %)
rUrease (1.0 mg/mL) CT-X (0.5 mg/mL)	PLGpS	7.35 ug/mg (N/D %) ⁴	rUrease = 4.5 ug/mg (53.0 %) CT-X = 2.5 ug/mg (59.0 %)
rUrease (1.0 mg/mL) PCPP (1.25 mg/mL)	PLGpS	5.97 ug/mg (67.2 %)	3.3 ug/mg (37.1 %) ⁵

- 1) AAA values are averages of two independent measurements of protein encapsulated within microparticles, as described in Example 6.
- polyclonal ELISA (capture sandwich antigen assay) conducted on microparticle hydrosylates, as described in Example 6. Average of 4 determinations.
- 3) Encapsulation efficiency (Encaps. Eff.) calculated as follows: total mass of protein recovered X 100% total amount of protein used
- 4) N/D = not determinable by AAA as total protein is actually a combination of rUrease and CT-X.
- 5) ELISA determination in the presence of PCPP was highly variable.

Table 8 Flu (trivalent) Formulations Examined by SRID's

Flu Samples conc. = 265 ug/mL	Solvent/Solution examined	Sonicate (30 sec)	Additives (quantity)	SRID A/Texas:B/Harbin:A/Johannes (ug/mL)
Entry #1	none	no	-	20.25 : 20.6 : 21.42
Entry #2	none	yes	-	21.73 : 19.8 : 23.05
Entry #3	EtOAc	yes		15.21 : N/D : 16.73
Entry #4	DCM	yes	-	17.28 : N/D : 20.54
Entry #5	EtOAc	yes	BAΥ (20 μg)	13.04 : 16.8 : 18.63
Entry #6	EtOAc	yes	DC-Chol (20 μg)	16.54 : 14.4 : 18.79
Entry #7	DCM	yes	BAΥ (20 μg)	18.44 : N/D : N/L
Entry #8	DCM	yes	DC-Chol (20 μg)	18.55 : N/D : N/L

N/D = not determined (below detection limit of $\sim 10~\mu g$ /mL). N/L = test failed or was not reproducible under experimental conditions.

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CLAIMS

What we claim is:

1. A biodegradable, biocompatible polymer having a backbone of the general formula:

wherein:

R₁, R₂, R₃, R₄, and R₅ are selected independently and are selected from H, linear or branched alkyl groups;
R₆ is selected from H, an amine protecting group, a spacer molecule or a biologically active species;
X is selected from an O or S group; and
x and y are integers.

- 2. The polymer of claim 1 wherein x and y are integers of values such that the polymer is comprised at least about 95% of α -hydroxy acid residues.
- 3. The polymer of claim 1 which is derived by copolymerization of monomers comprising at least one α -hydroxy acid and at least one pseudo- α -amino acid.
- 4. The polymer of claim 3, wherein the at least one α -hydroxy acid has the formula $R_1R_2COHCO_2H$, wherein the R_1 and R_2 groups are H, linear or branched alkyl units, the alkyl unit being represented by the formula C_nH_{2n+1} , where n = integer of about 1 to 10.
- 5. The polymer of claim 4, wherein the α -hydroxy

acids comprise a mixture of α -hydroxy acids, one of said mixture of α -hydroxy acids having R_1 and R_2 groups which are hydrogen and the other of said mixture of α -hydroxy acids having an R_1 group which is CH_3 and R_2 group which is H.

- 6. The polymer of claim 3, wherein the at least one pseudo- α -amino acid has the formula $R_5CHNHR_6CO_2H$, wherein the R_5 group is a hydroxyl methyl or methyl thiol group and R_6 is an amine protecting group.
- polymer of claim 6, wherein the protecting group is selected from the group consisting carbobenzyloxy (CBZ or benzyl Z), (Bn), methoxybenzyl (MeOBn), benzyloxymethoxy (BOM), butyloxycarbonyl (t-BOC) and [9-fluorenylmethyl oxy]carbonyl (FMOC).
- 8. The polymer of claim 3, wherein the at least one α -hydroxy acid is selected from the group consisting of L-lactic acid, D,L-lactic acid, glycolic acid, hydroxy valeric acid and hydroxybutyric acid.
- 9. The polymer of claim 3, wherein the at least one pseudo- α -amino acid is derived from serine.
- 10. The polymer of claim 1, wherein said polymer is poly-D,L-lactide-co-glycolide-co-pseudo-Z-serine ester (PLGpZS).
- 11. The polymer of claim 1, wherein said polymer is poly-p,L-lactide-co-glycolide-co-pseudo-serine ester(PLGpS).
- 12. The polymer of claim 1, wherein R_6 is at least one biologically active species.

- 13. The polymer of claim 12, wherein R_6 is at least one spacer molecule to which at least one biologically active species is coupled.
- 14. The polymer of claim 13, wherein the spacer molecule is selected from α -hydroxy acids represented by the formula $R_7R_8COHCO_2H$, wherein the R_7 or R_8 groups are independently H, linear or branched alkyl units and pseudo- α -amino acids represented by the formula $R_9CHNHR_{10}CO_2H$, wherein the R_9 group is a hydroxyl methyl or methyl thiol group and R_{10} is an amine protecting group.
- 15. The polymer of claim 12, wherein the at least one biologically active species is selected from the group consisting of cell bioadhesion groups, macrophage stimulators, poly amino acids and polyethylene glycol.
- 16. The polymer of claim 1 having a molecular weight of about 5000 to about 40,000 daltons.
- 17. A process for making a biodegradable, biocompatible polyester, which comprises copolymerizing at least one α -hydroxy acid monomer and at least one pseudo- α -amino acid monomer.
- 18. A process for making a biodegrable, biocompatible polymer of the formula shown in claim 1, which comprises;
- forming a mixture of monomers comprising at least one α -hydroxy acid and at least one pseudo- α -amino acid having an amine protecting group with an organic solvent solution of an esterification catalyst under inert atmospheric conditions;
 - copolymerizing said monomers; and
 - isolating the resultant polymer.

- 19. The process of claim 18, wherein the polymer formed is deprotected by solid phase catalytic reduction or acid catalysis.
- 20. The process of claim 19, wherein said deprotection is by acid catalysis in the presence of hydrogen bromide in acetic acid solution.
- 21. The process of claim 18, wherein said catalyst is stannous 2-ethylhexanoate.
- 22. The process of claim 18, wherein said polymerization is carried out at a temperature of about 120°C for about 28 hours.
- 23. The process of claim 18, wherein said organic solvent is anhydrous chloroform.
- 24. The process of claim 18, wherein said process further comprises forming the polymer into a film.
- 25. The process of claim 18, wherein said process further comprises forming the polymer into microparticles.
- 26. A particulate carrier for delivery of biologically active materials to a host, said carrier comprising a polymer having a backbone of the general formula:

wherein:

 R_1 , R_2 , R_3 , R_4 and R_5 are selected independently and are selected from H, linear or branched alkyl groups;

 R_6 is selected from H, an amine protecting group, a spacer molecule or a biologically active species;

- X is selected from an O or S group; and
- x and y are integers.
- 27. The particulate carrier of claim 26, wherein said carrier has a particle size of about 1 to 50 μM_{\odot}
- 28. The particulate carrier of claim 27 wherein said polymer has a molecule weight of about 5,000 to about 40,000 daltons.
- 29. The particulate carrier of claim 28 wherein at least about 95% of the polymer is comprised of α -hydroxy acid residues.
- 30. A composition comprising the particulate carrier of claim 26 and at least one biologically active material entrapped therein.
- 31. A composition comprising the particulate carrier of claim 26 and at least one biologically active material physically mixed therewith.
- 32. The composition of claim 30 or 31, wherein said at least one biologically active material is capable of eliciting an immune response.
- 33. The composition of claim 32, wherein said at least one biologically active material comprises at least one Haemophilus influenzae protein.
- 34. The composition of claim 33, wherein said Haemophilus influenzae protein is selected from the group consisting of a non-proteolytic Hin-47 analog, D15, P1, P2, P6 and mixtures thereof.
- 35. The composition of claim 32, wherein said at least one biologically active material comprises at least one

influenza virus or influenza virus protein.

- 36. The composition of claim 35 wherein said influenza virus comprises a multivalent influenza virus vaccine.
- 37. The composition of claim 35 wherein said influenza virus comprises a monovalent influenza virus vaccine.
- 38. The composition of claim 35 wherein said influenza virus protein comprises a influenza virus monovalent protein vaccine.
- 39. The composition of claim 32 wherein said at least one biologically active material comprises at least one *Moraxella catarrhalis* protein.
- 40. The composition of claim 39 wherein said at least one *M. catarrhalis* protein is a Tbp2 protein of *M. Catarrhalis*.
- 41. The composition of claim 32 wherein said at least one biologically active material comprises at least one Helicobacter pylori protein.
- 42. The composition of 41 wherein said *H. pylori* protein comprises Urease.
- 43. The composition of claim 26 further comprising at least one adjuvant entrapped therein.
- 44. The composition of claim 26 further comprising at least one adjuvant mixed therewith.
- 45. The composition of claim 43 or 44, wherein said adjuvant is selected from the group consisting of lipopeptides, mineral salts, lipids, glycolipids, carbohydrates and combinations, derivatives and mixtures thereof.
- 46. The composition of claim 45 wherein said adjuvant is an organic solvent soluble adjuvant.

- 47. The composition of claim 46 wherein said adjuvant is selected from the group consisting of BAY R1-005, tripalmitoyl cysteine and DC-chol.
- 48. The composition of claim 45 wherein said adjuvant is a water soluble adjuvant.
- 49. The composition of claim 48 wherein said adjuvant is a polymeric water soluble adjuvant.
- 50. The composition of claim 49 wherein said polymer adjuvant is PCPP.
- 51. The composition of claim 49 wherein said water soluble adjuvant is a mucosal adjuvant.
- 52. The composition of claim 51 wherein said mucosal adjuvant is CT-X.
- 53. The composition of claim 51 wherein said mucosal adjuvant is LT.
- 54. The composition of claim 30 or 31, wherein said carrier comprises a matrix having a particle size of about 1 to 50 uM.
- 55. A process for making a particulate carrier for the delivery of at least one biologically active material to a host, comprising the steps of:
- (a) mixing an organic solvent phase comprising an α -hydroxy acid polymer or copolymer with an aqueous composition comprising dispersed or dissolved biologically active material to form a first water-in-oil emulsion:
- (b) dispersing the first water-in-oil emulsion into an aqueous detergent phase to form a second water-in-oil-in-water double emulsion;
 - (c) removing water from the second double

emulsion to form microspheres; and

- (d) collecting the microspheres and having said biological material entrapped therein.
- 56. The process of claim 55, wherein said polymer is selected from the group consisting of poly-D-, L-lactide-co-glycolide (PLG), poly-D,L-lactide-co-glycolide-co-pseudo-Z-serine ester (PLGpZS) and poly-D,L-lactide-co-glycolide-co-pseudo-serine ester (PLGpS).
- 57. The process of claim 55, wherein said organic solvent is selected from the group consisting of dichloromethane, ethyl acetate, acetone and mixtures thereof.
- 58. The process of claim 58, wherein said aqueous detergent phase comprises at least one non-ionic emulsion stabilizer.
- 59. The process of claim 58, wherein said at least one non-ionic emulsion stabilizer is selected from the group consisting of poly vinyl alcohol, methyl cellulose and Triton X-100.
- 60. The process of claim 55, wherein said first water-in-oil emulsion additionally comprises at least one organic solvent soluble adjuvant.
- 61. The process of claim 60 wherein said organic solvent soluble adjuvant is lipophilic.
- 62. The process of claim 61, wherein said adjuvant is selected from the group consisting of BAY R1-005, tripalmitoyl cysteine and DC-Chol.
- 63. The process of claim 55 wherein said first water-in-oil emulsion additionally comprises at least one water soluble adjuvant.

- 64. The process of claim 63 wherein said water soluble adjuvant is a polymer water soluble adjuvant.
- 65. The process of claim 64 wherein said polymeric water soluble adjuvant is PCPP.
- 66. The process of claim 62 wherein said water soluble adjuvant is a mucosal adjuvant.
- 67. The process of claim 63 wherein said mucosal adjuvant is CT-X.
- 68. The process of claim 66 wherein said mucosal adjuvant is LT.
- 69. The process of claim 55, wherein said first water-in-oil emulsion additionally comprises at least one surfactant.
- 70. The process of claim 69, wherein said surfactant is selected from the group consisting of sucrose, mannose, trehalose and gelatin.
- 71. An immunogenic composition comprising the particulate carrier of claim 26, an immunogen, and a physiologically acceptable carrier therefor.
- 72. A method of producing an immune response in a host comprising administering the immunogenic composition of claim 71 to said host.
- 73. The method of claim 72, wherein said composition is administered mucosally or parenterally.
- 74. The method of claim 72, wherein said immune response is an antibody response.
- 75. The method of claim 74, wherein said antibody response is a local or serum antibody response.

Poly-D,L-Lactide-co-Glycolide-co-pseudo-L-Z-Serine Ester (PLGpZS)

Poly-D,L-Lactide-co-Glycolide-co-pseudo-L-Serine Ester (PLGpS)

FIG.1

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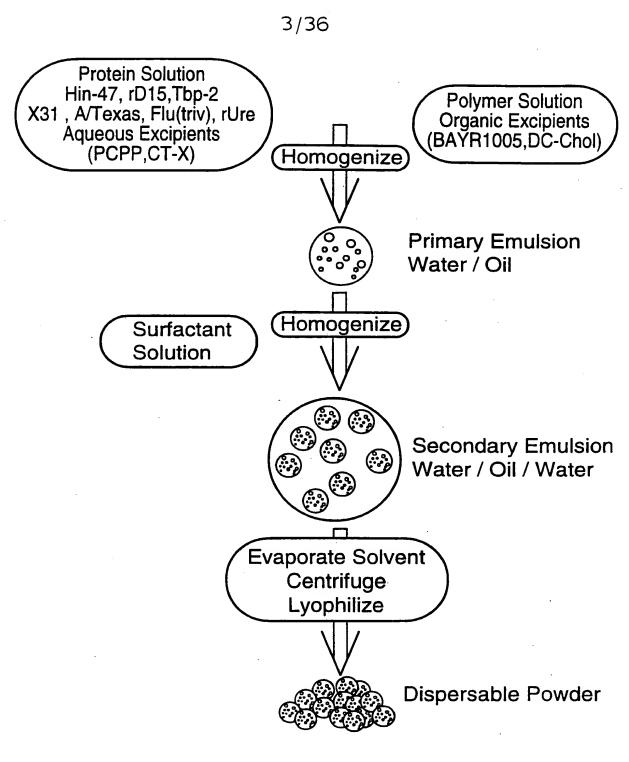


FIG.3

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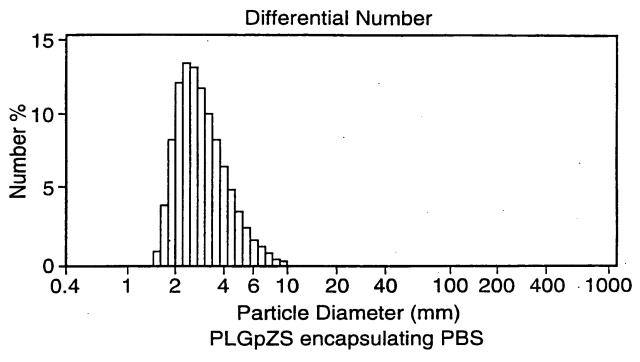
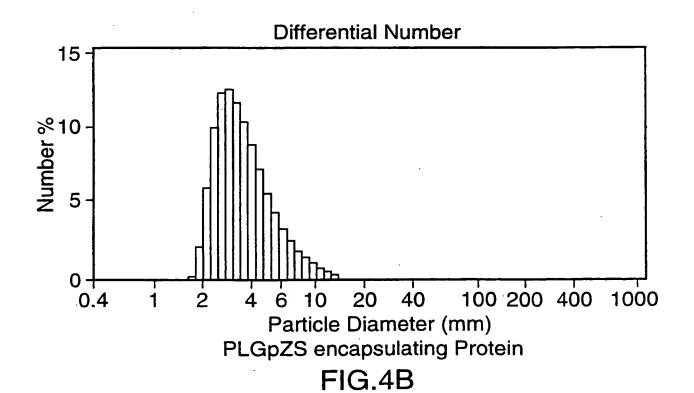
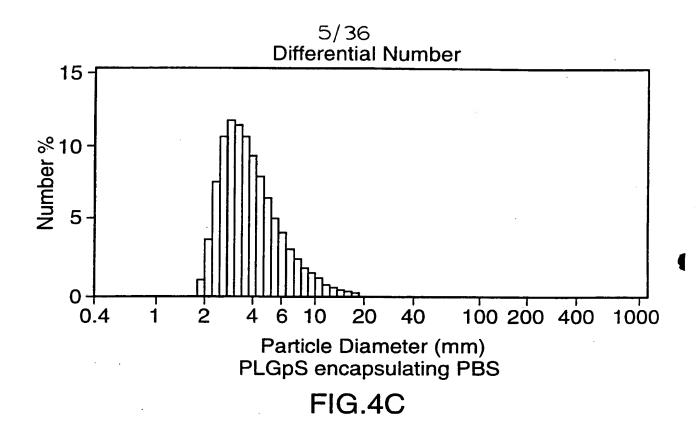


FIG.4A





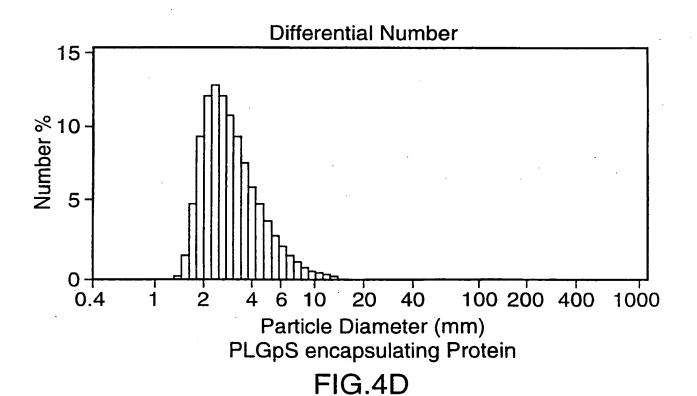
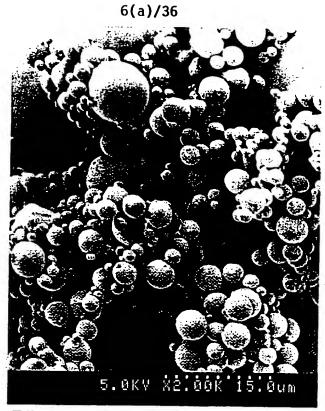
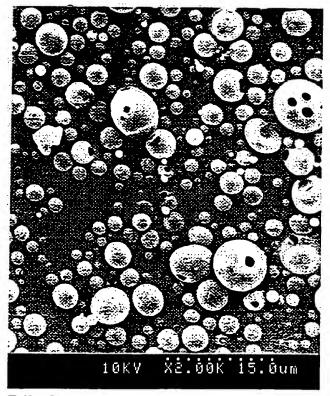


FIG.5A



PLGpZS encapsulating PBS

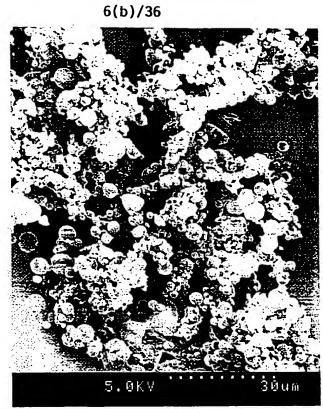
FIG.5B



PLGpS encapsulating PBS

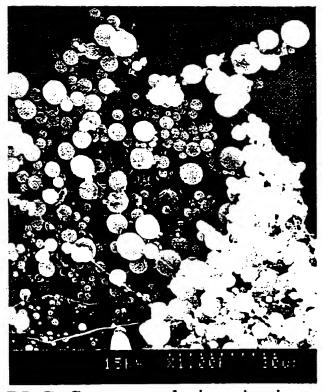
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FIG.6A



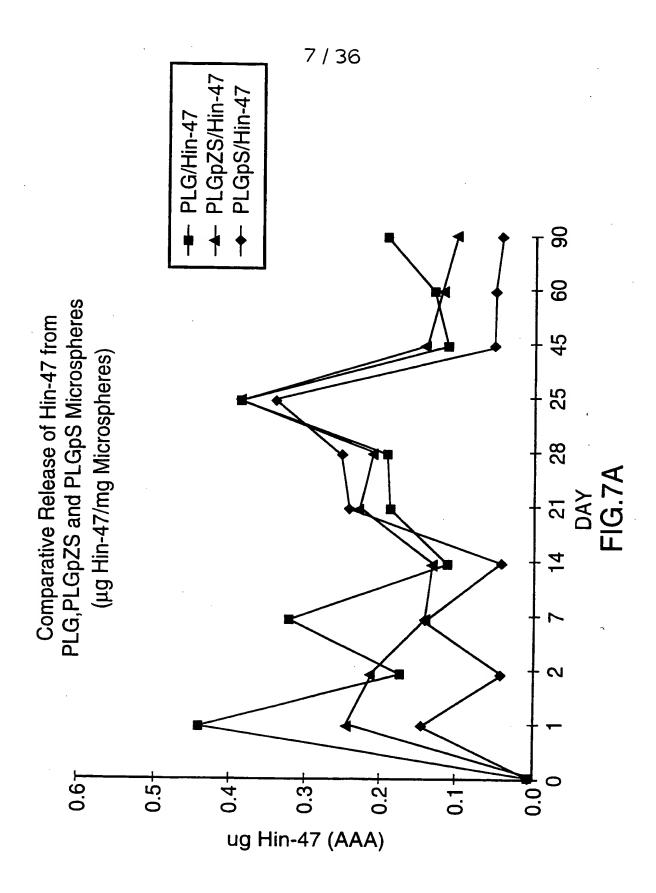
PLGpZS encapsulating Antigen

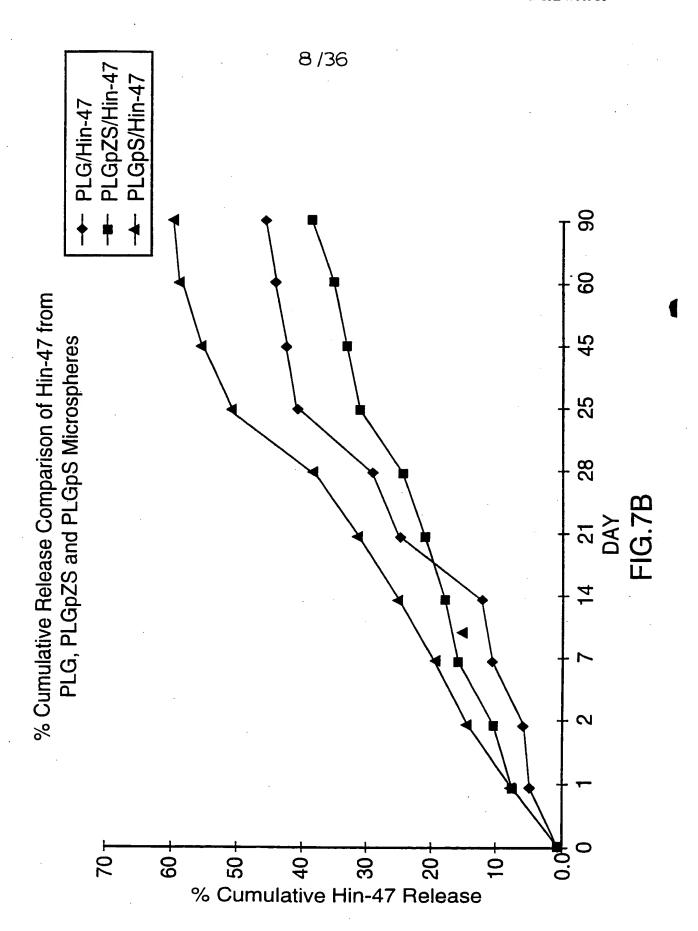
FIG.6B



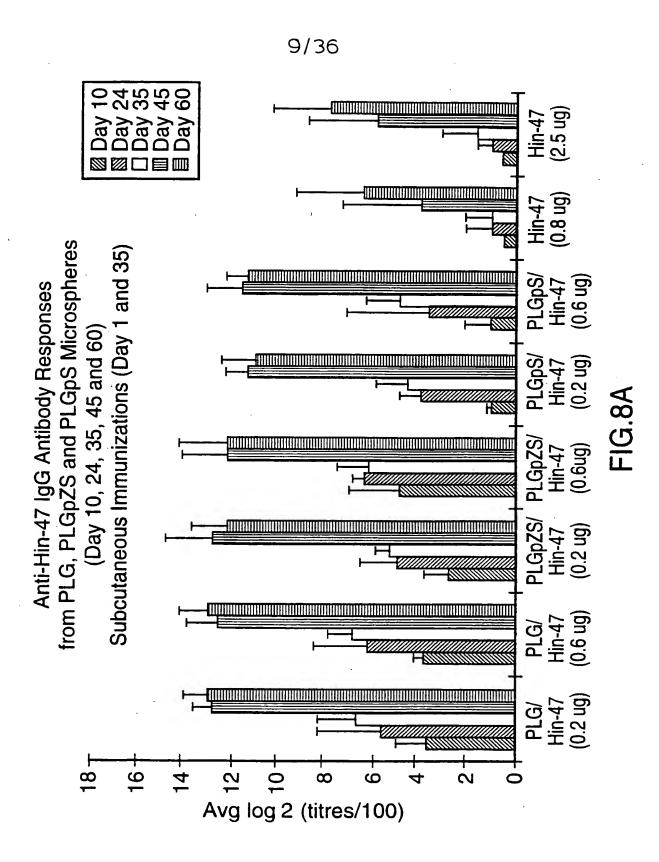
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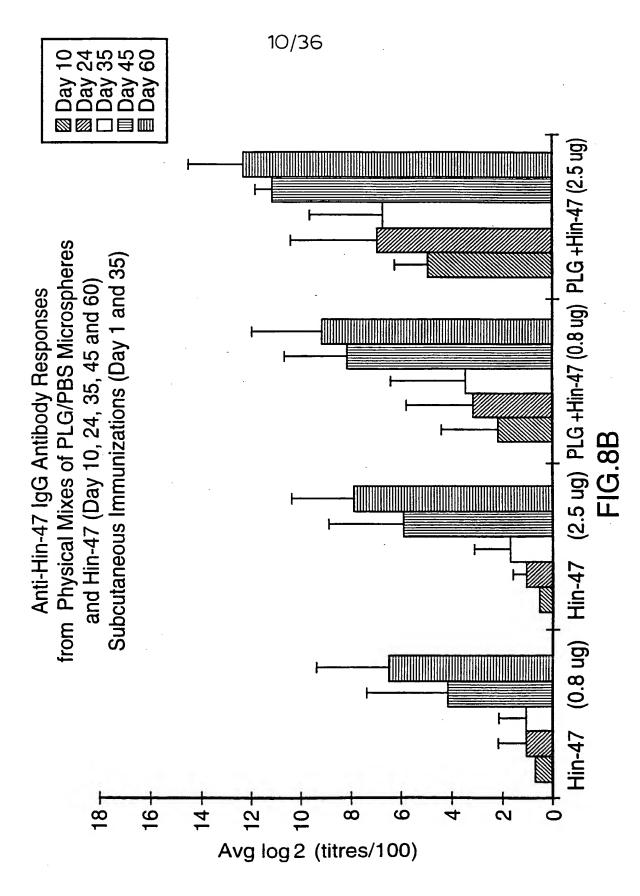




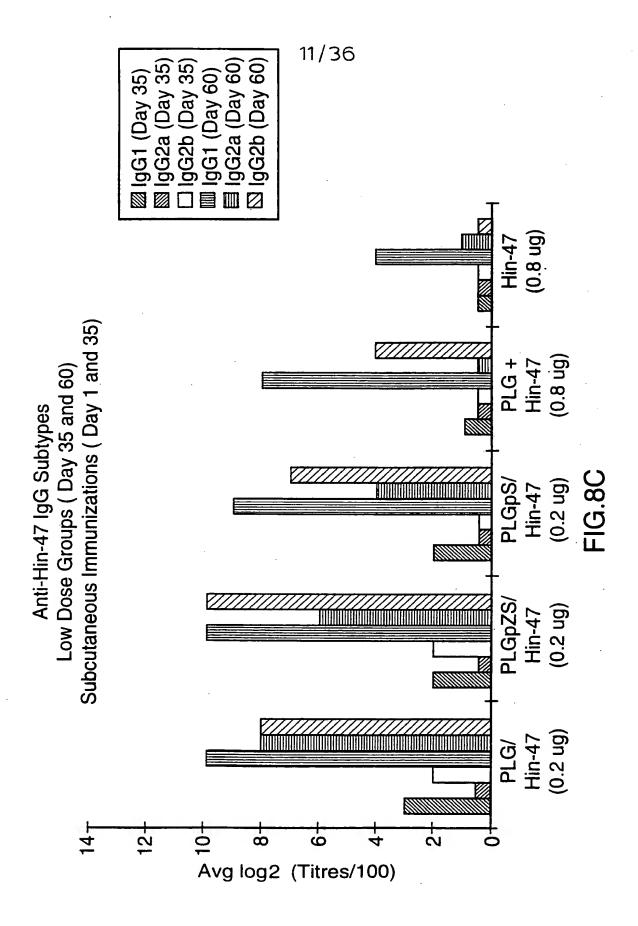
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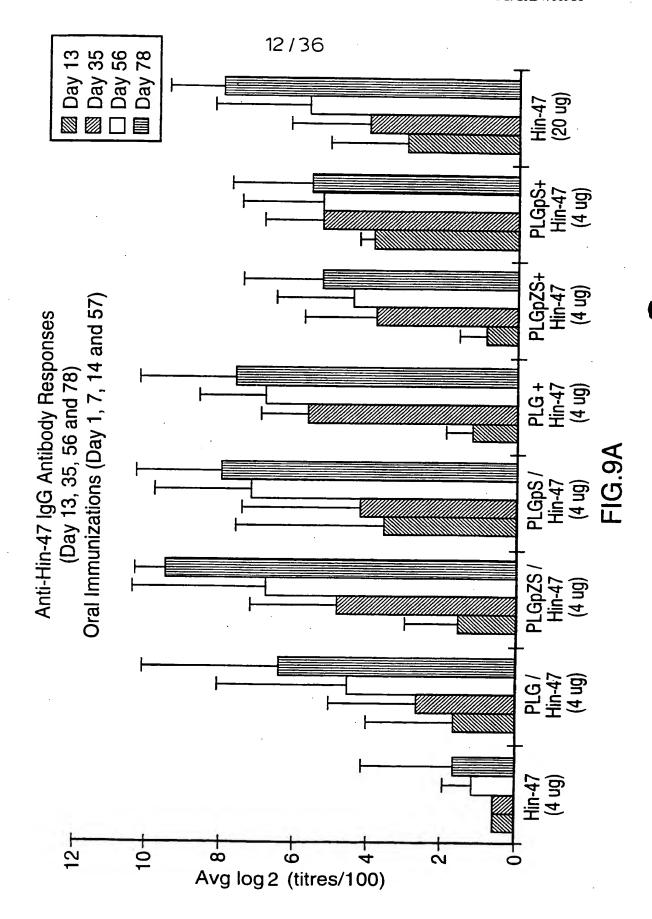


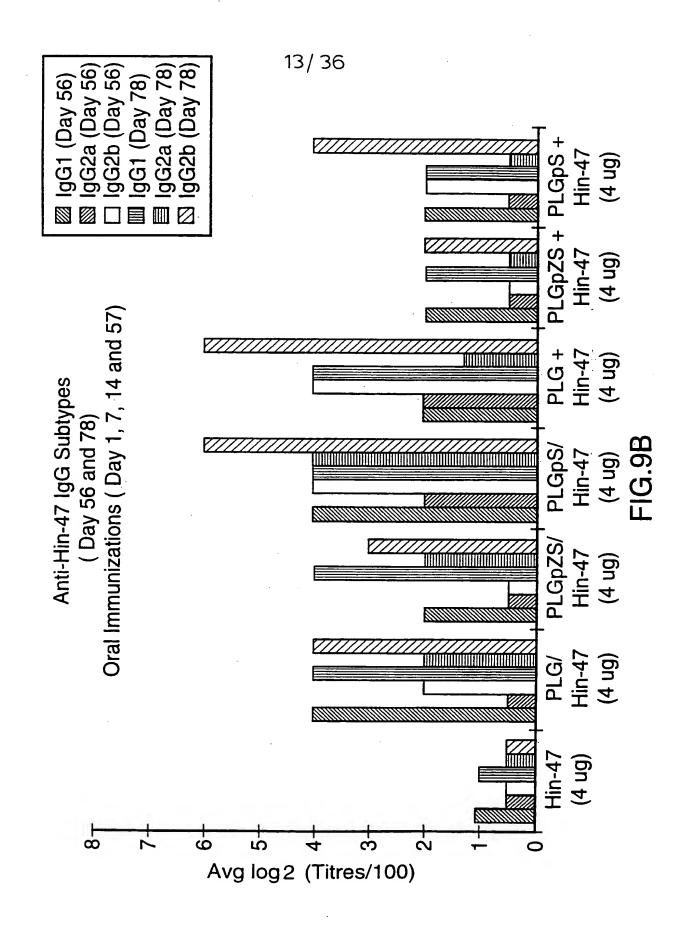
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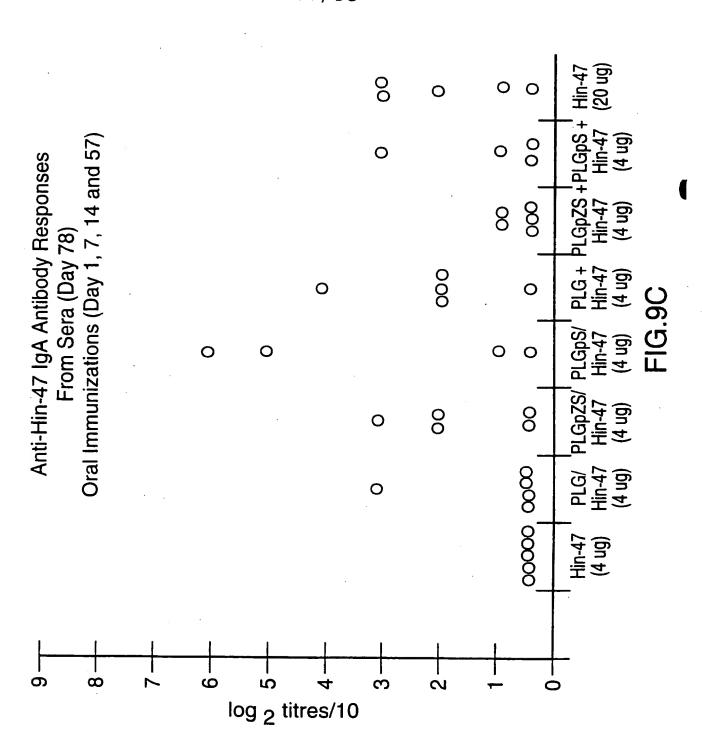




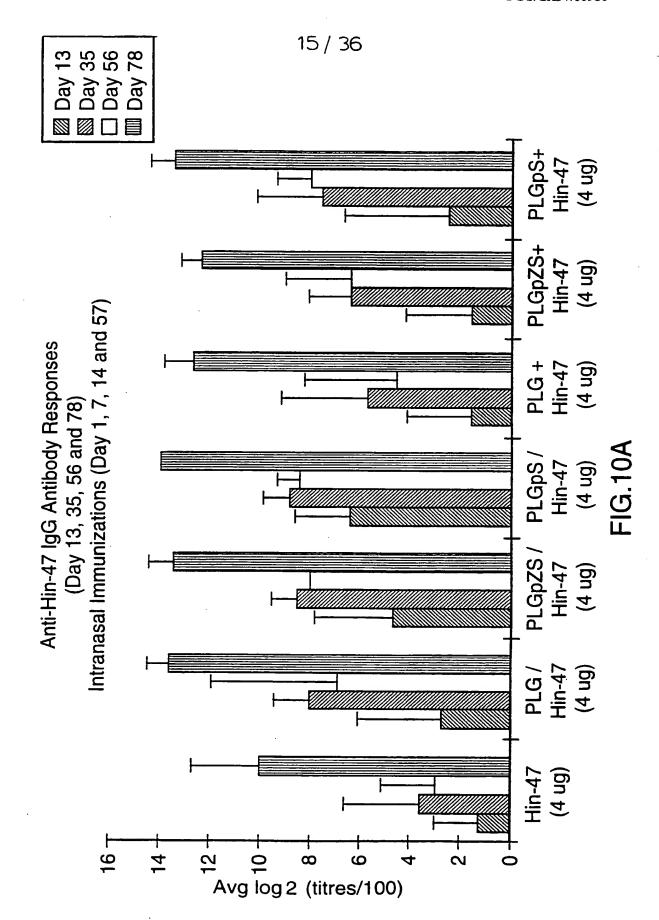


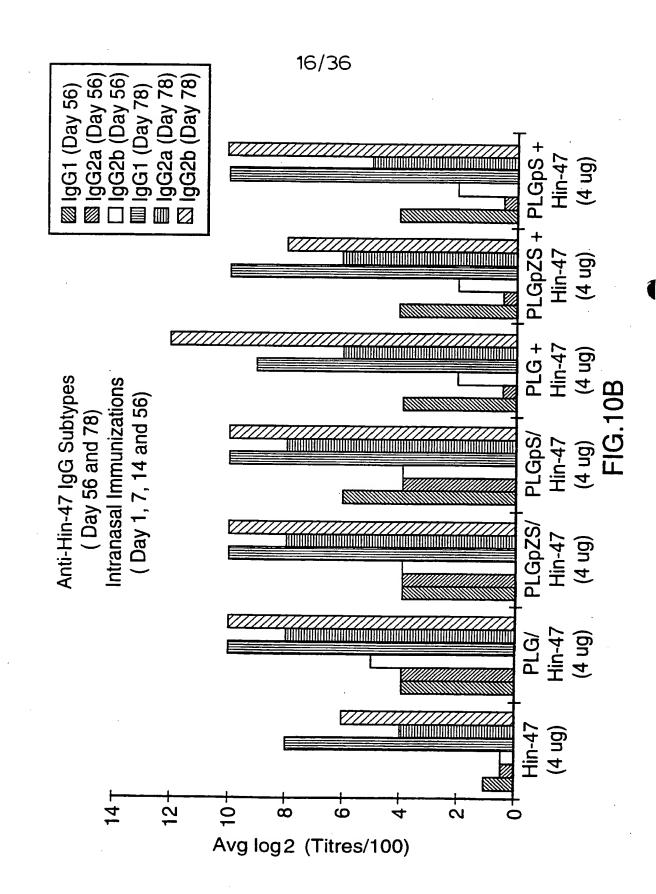
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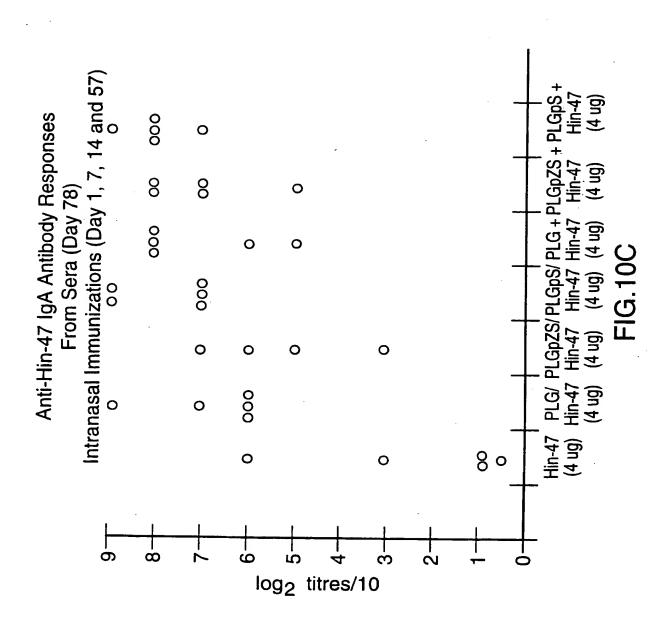


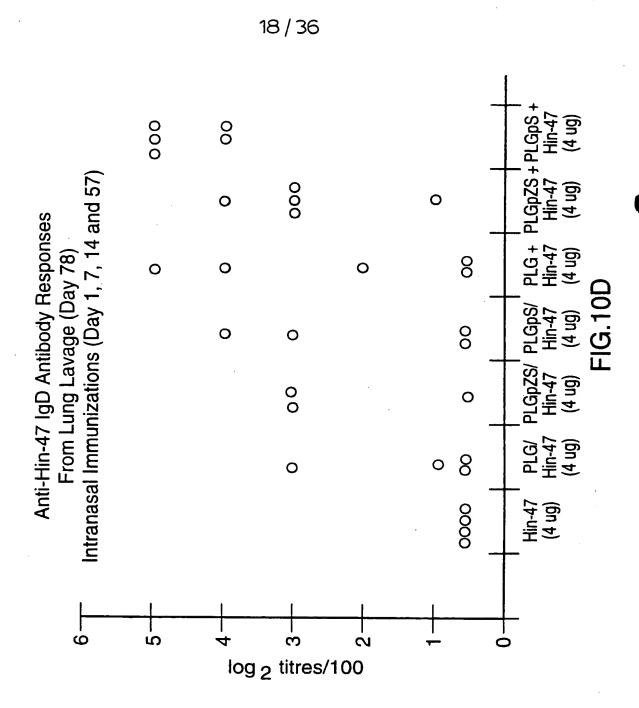
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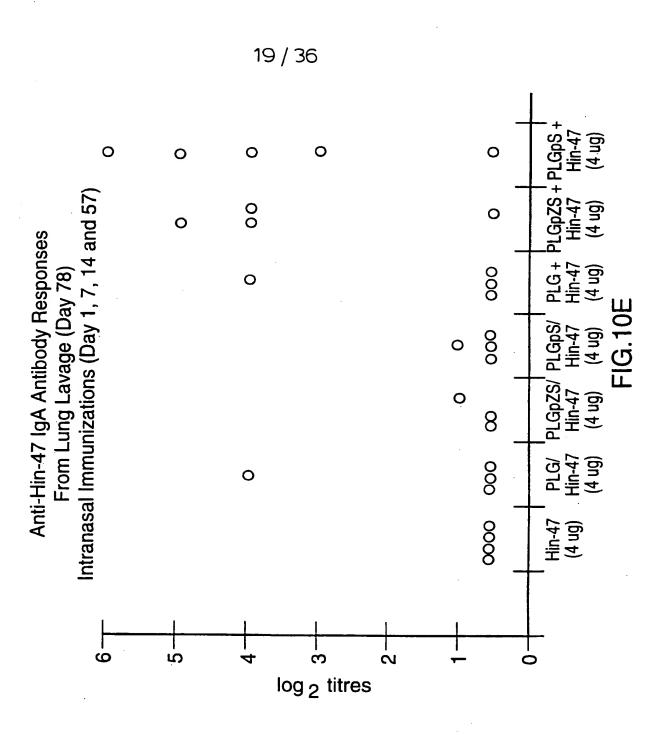


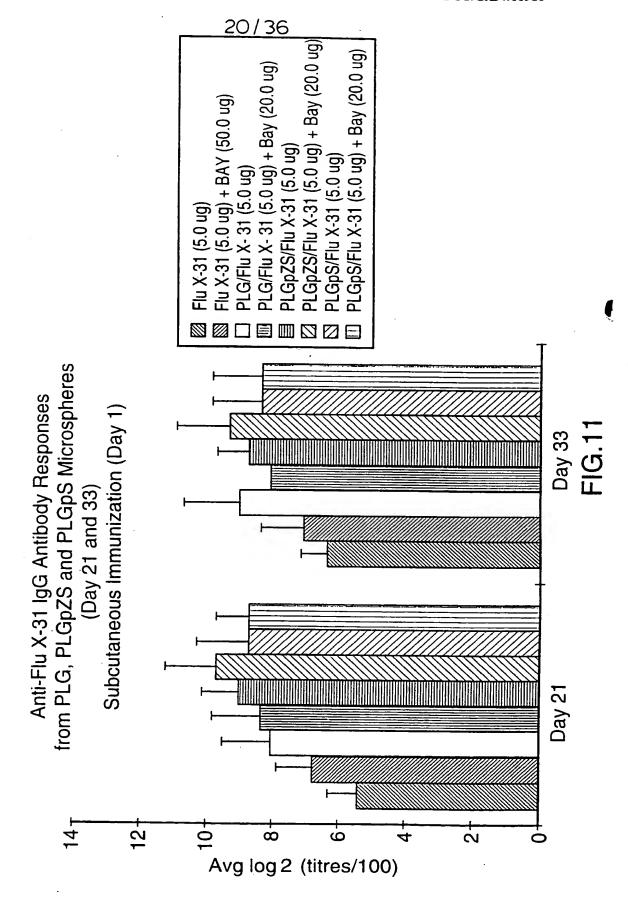


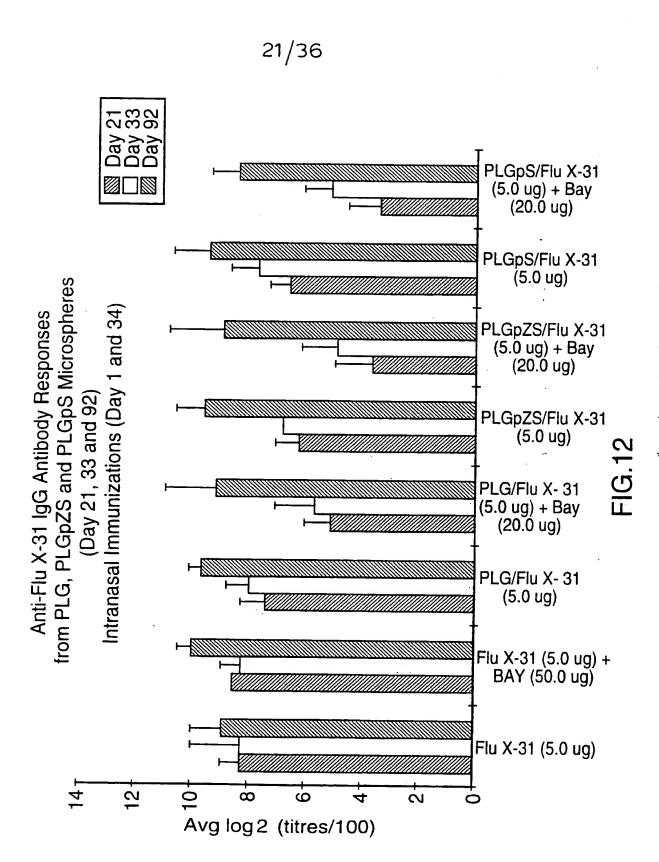
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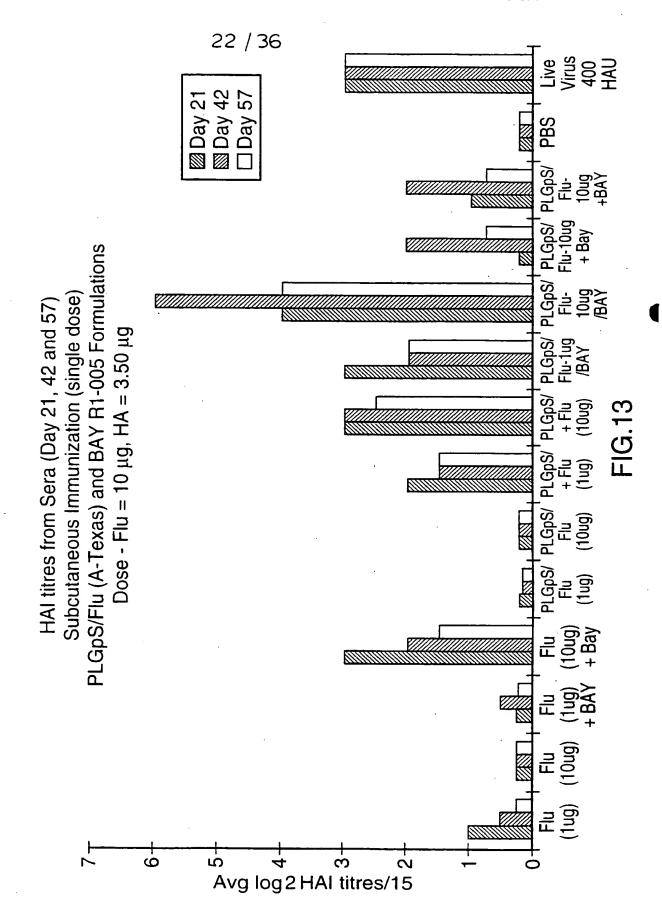


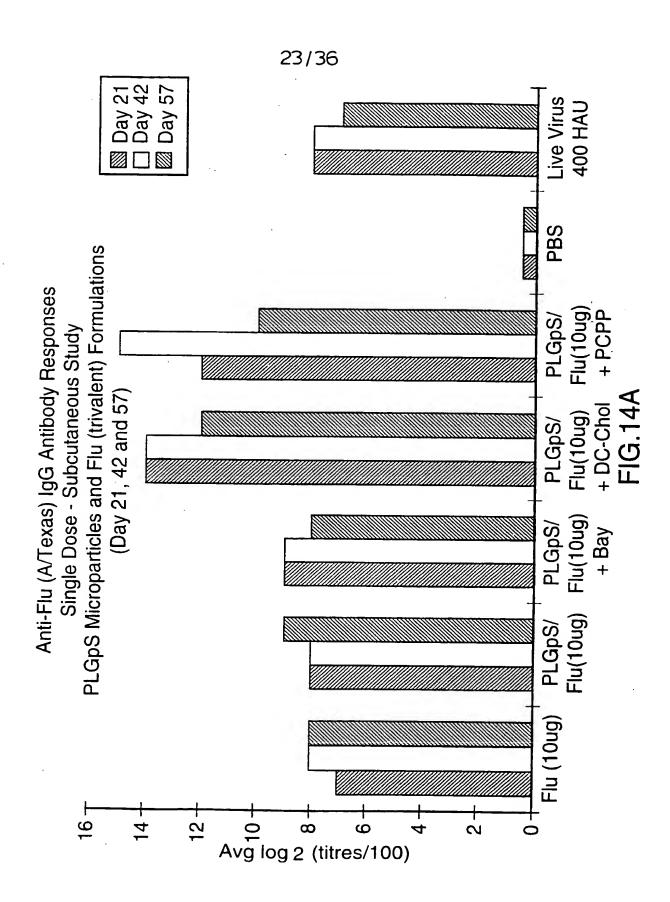


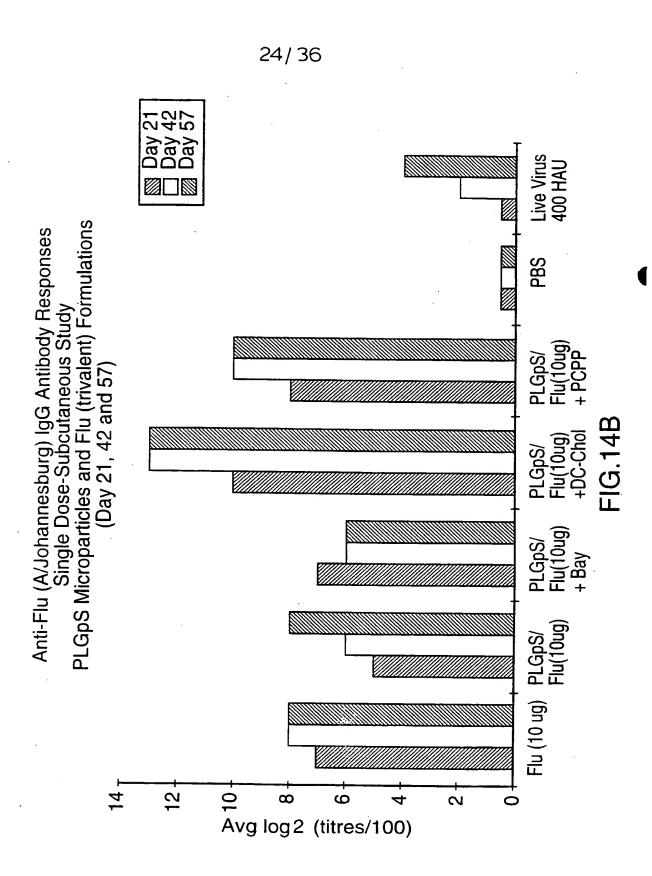


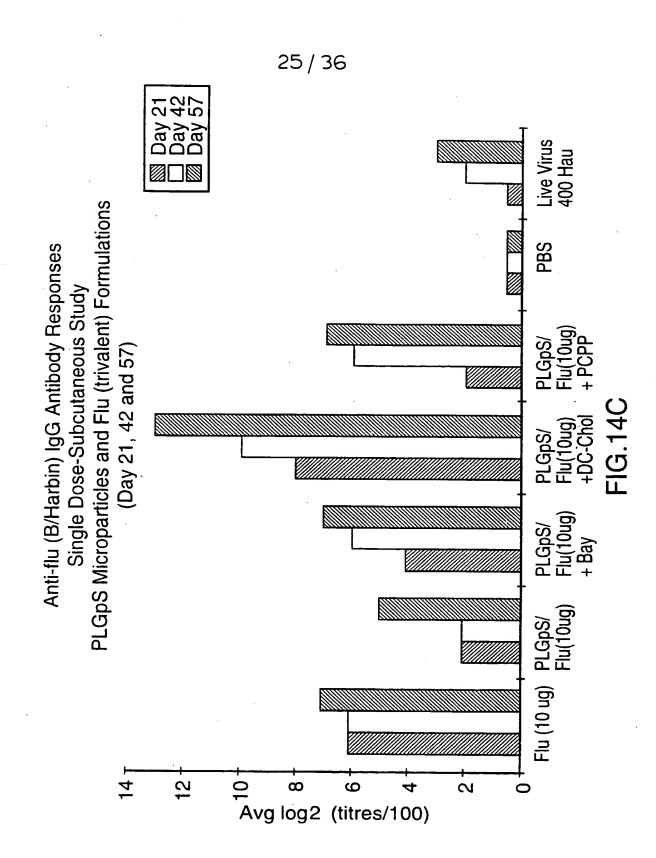


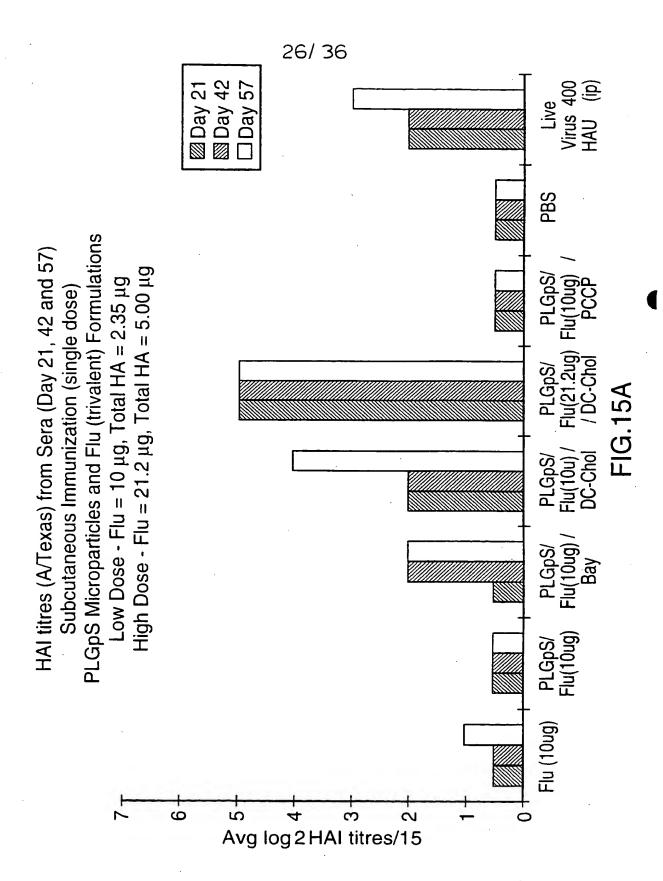


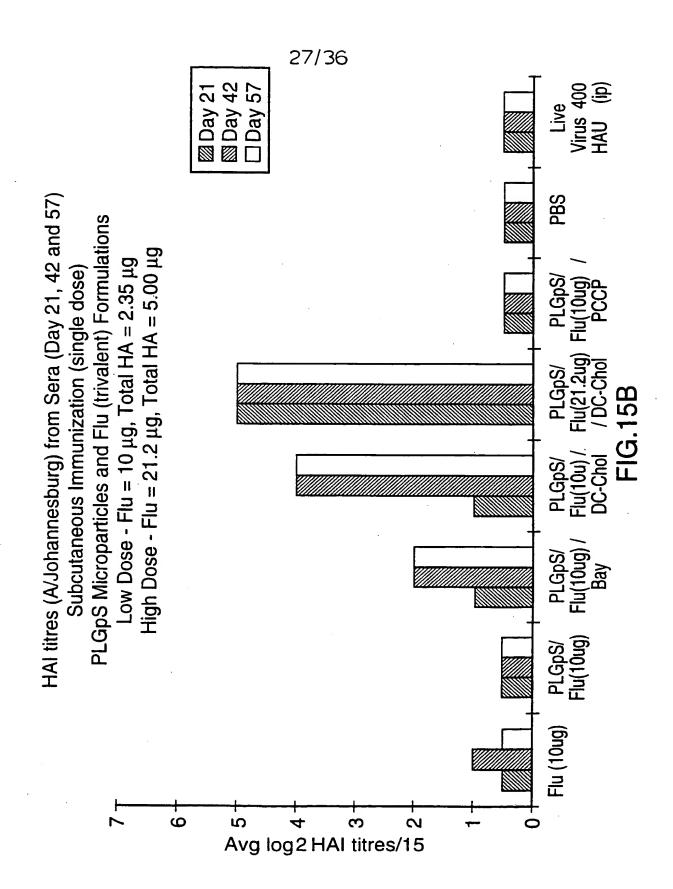


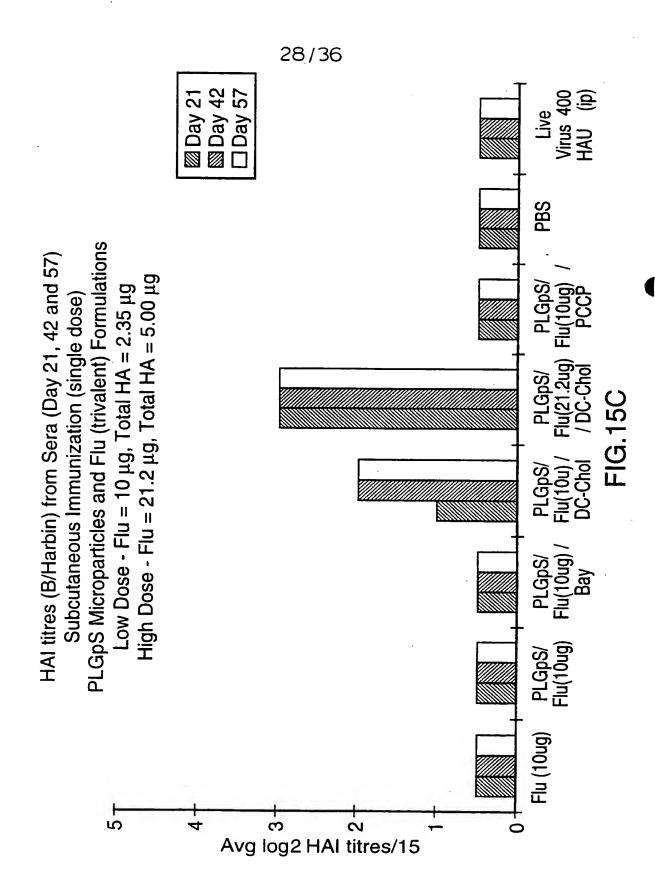


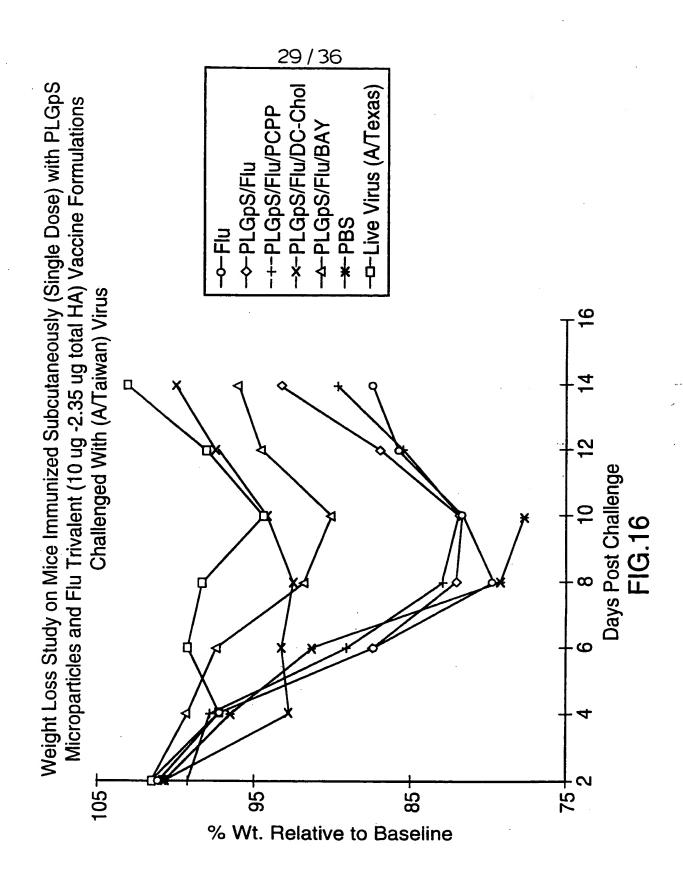


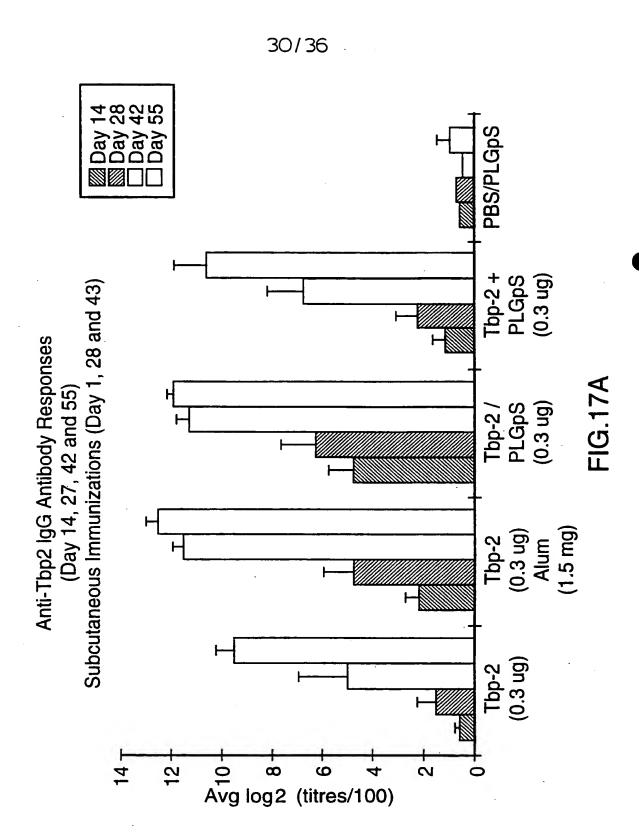


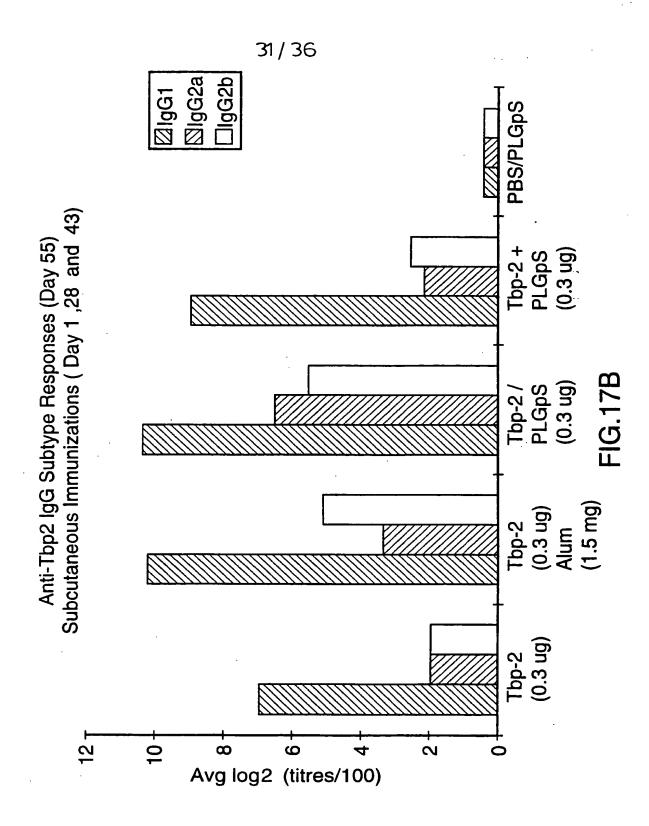


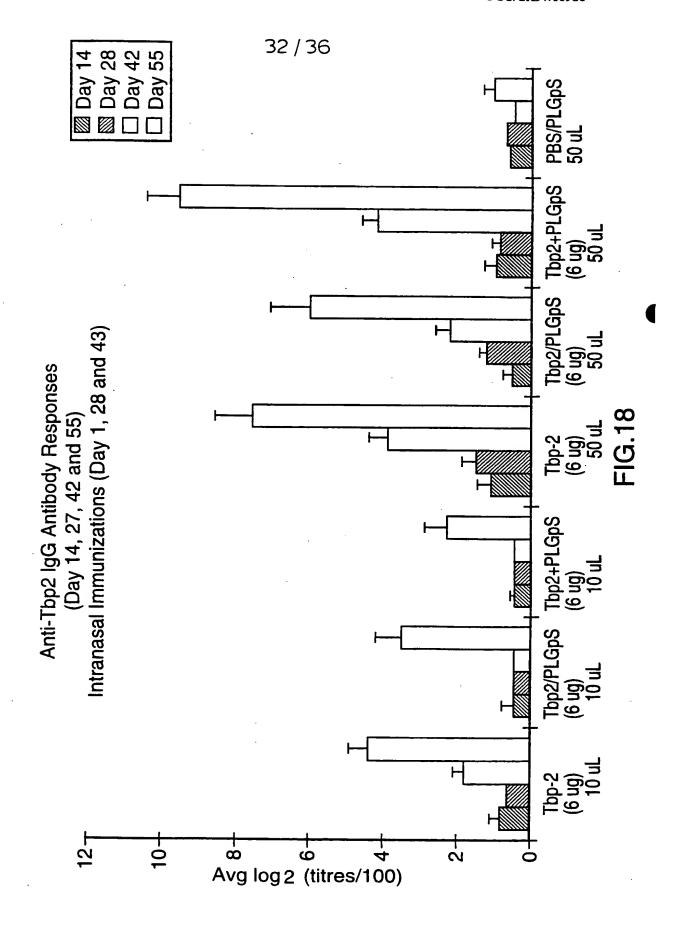


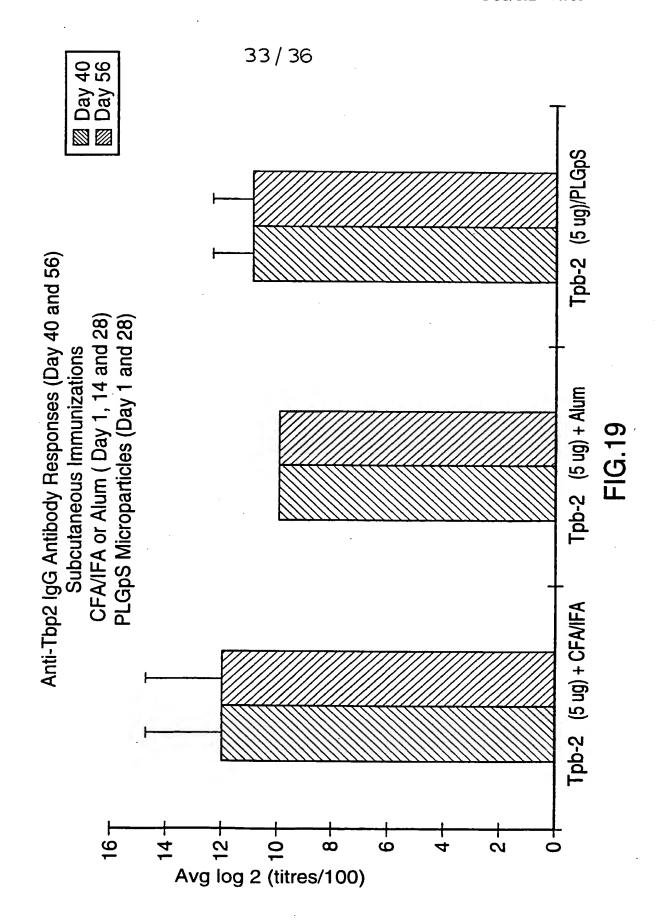


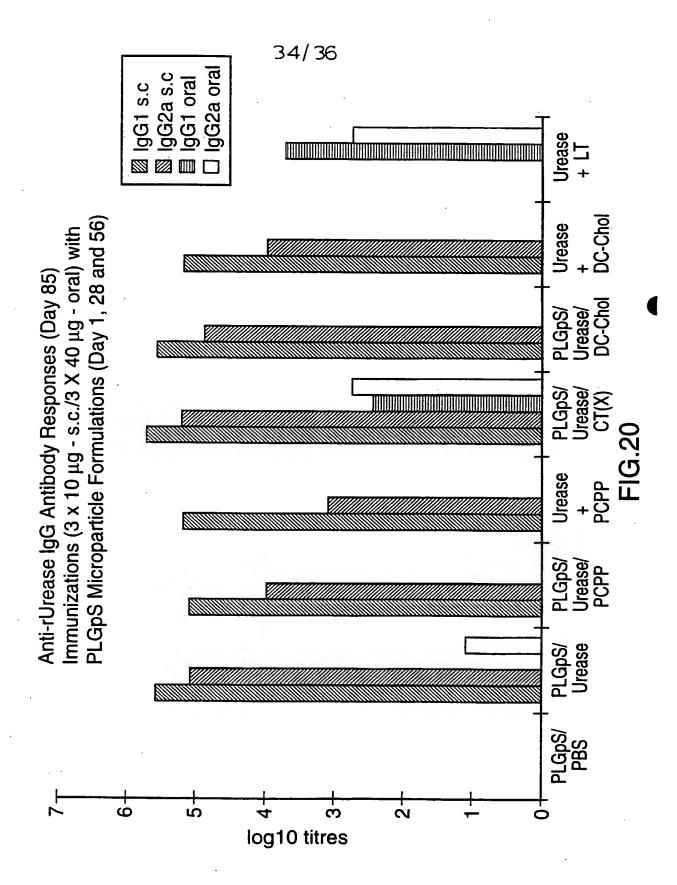


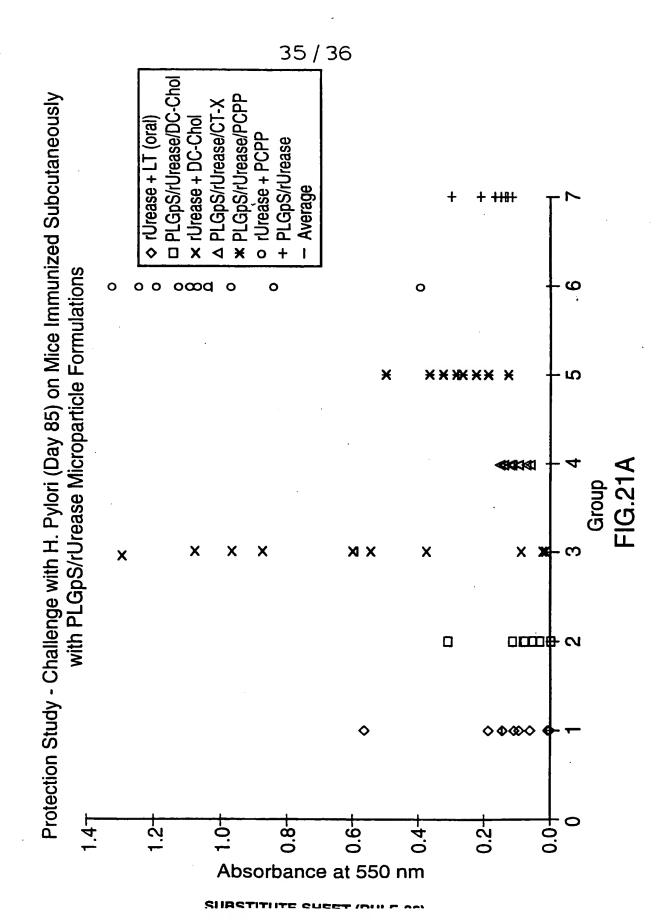


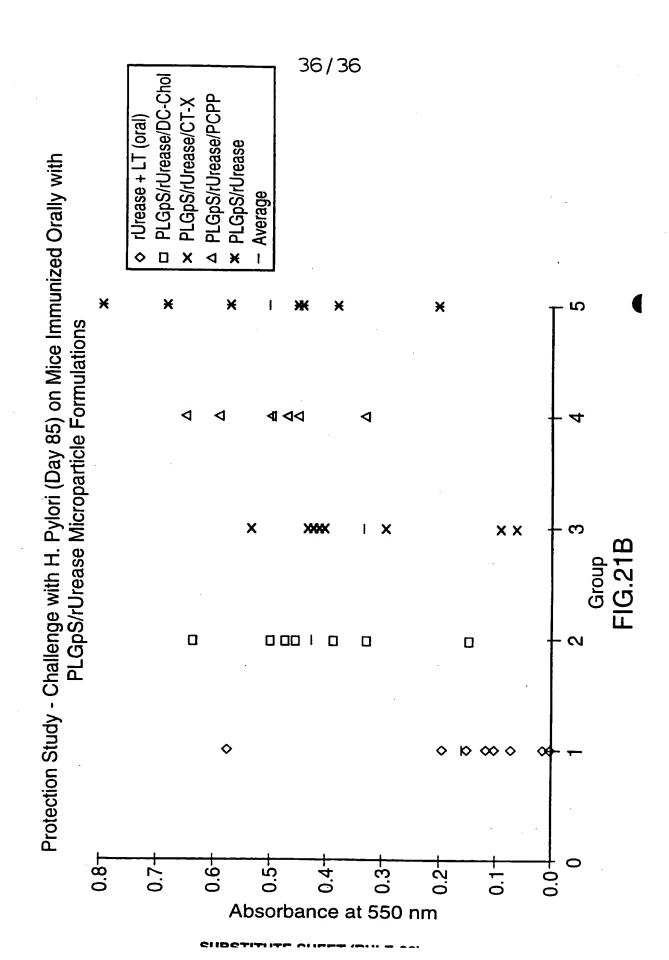












INTERNATIONAL SEARCH REPORT

Int ional Application No

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A. CLASSIFICATION IPC 6 COSC	of SUBJECT MATTER 363/685 C08G75/26 A61K9/	/ 16				
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B. FIELDS SEARCHED						
Minimum documentation IPC 6 COSG	on searched (classification system followed by classific A61K	cation symbols)				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched						
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)						
C. DOCUMENTS CON	SIDERED TO BE RELEVANT					
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Int Honal Application No PCT/CA 97/00980

						
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